ISOLATION AND CHARACTERIZATION OF TAXANES AND OTHER COMPOUNDS FROM VARIOUS SPECIES OF *TAXUS*

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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This work is dedicated to the memory of Dr. Koppaka V. Rao, an extraordinary scientist, dedicated teacher and very dear friend. I feel blessed to have known and worked with him.

ACKNOWLEDGMENTS

Much of the work in this dissertation was done with the guidance and expertise of the late Dr. K. V. Rao, who passed away on February 20, 1998. Professor Rao and I had known each other since 1981 and I am grateful to him for encouraging me to return for graduate studies and for his true friendship with me. I would also like to thank his wife and children for their much appreciated support, friendship and encouragement.

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Chairman: Koppaka V. Rao
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Major Department: Medicinal Chemistry

Taxol is a promising antineoplastic agent originally reported in 1971 by Wani and Wall, isolated from the bark of the Pacific yew (*Taxus brevifolia).* Intensive research in the last decade has demonstrated that this drug possesses exceptional activity in the treatment of many difficult types of cancer.

From the beginning taxol has proven to be a difficult compound to obtain, with very low yields and a highly complex structure with many chiral centers and sensitive moieties. Originally obtained from the bark of a very slow growing tree, the possibility of growing various *Taxus* (yew) species under hydroponic conditions has been investigated in this project.

One local variety, known as *Taxus floridana* (Florida yew) was found to grow well and produce taxol and other useful taxanes. During initial investigations a simple and elegant method for the isolation of taxol using reverse phase bonded silica was developed. Generous funding by the University of Florida Division of Sponsored

Research made possible the construction of a pilot plant scale facility where these isolation methods were successfully implemented.

Excellent yields and the isolation of many related taxanes have proven that this method is superior to currently approved processes used in the production of taxol. The failure of other researchers to employ bonded silica gel for preparative columns in the past may reflect experiences with analytical columns, but this method has proven to be quite exceptional and should be employed extensively.

This dissertation covers many crystalline and non-crystalline compounds isolated and characterized as a result of this project. Some results from the application of this technique for the isolation of taxanes from the needles of *Taxus brevifolia, Taxus* x *media* cultivar Hicksii, and *T. floridana* are presented. Similar experiments on the bark and wood of *T. brevifolia* are also described.

CHAPTER 1
HISTORICAL OVERVIEW OF TAXUS

During the late 1950s, the National Cancer Institute initiated a program with the objective of discovering compounds from natural sources, which might prove useful in the treatment of various human cancers. In this program, plant samples from various parts of the world were collected and brought to participating laboratories, where the active principles were isolated, chemically characterized, and subjected to testing in various murine tumors.

It is in such a context that a sample of the bark of the Pacific yew (*Taxus brevifolia*, Nutt.) was extracted and the active principle called taxol* was reported by Wani and coworkers in 1971 (Wani *et al.* 1971). Although it exhibited potent cytotoxicity in some tumor assays, many unsuccessful lead compounds also are cytotoxic in these assays. Taxol did not appear exceptional, and the problems of low yield and poor solubility discouraged the pursuit of further research for many years. During the late 1970s, activity against B-16 melanoma in mice, and several human tumors grown in athymic mice was recognized (e.g. MX-1 human mammary xenograft). These activities rekindled interest in taxol as a candidate for cancer treatment, resulting in further studies and human clinical trials. In the ensuing years the pace of research into taxol and taxoids has increased dramatically.

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*Taxol® is a registered trademark since 1993 by Bristol Myers Squibb Co., with paclitaxel being the generic name. 'Taxol' will be used in this dissertation as the generic name, as this work was started before this change.

A study of its mode of action revealed that it blocked cell division at the cell cycle through its specific action on the G_2/M phase of the tubulin/ microtubule system. Unlike other antitumor drugs such as colchicine, vincristine and vinblastine, which act as tubulin poisons, taxol exhibited a novel mode of action (Schiff *et al.* 1979). Microtubules are involved in the formation of the mitotic spindle fibers necessary for the replication of DNA and are also integral building blocks within the cell wall. They are generated from a protein known as tubulin, and a dynamic equilibrium exists between tubulin and microtubules *in vivo*. In the presence of taxol, the polymerization of tubulin produces what are now known as oligo-microtubules. In contrast to the usual microtubules, which can be readily disassembled, these oligo-microtubules resist disassembly to tubulin, thereby preventing cell division (Horwitz, 1992).

Based on potent activity against important experimental tumors and its unique mode of action, interest in taxol became greatly enhanced, and it was approved for human Phase I clinical trials in the early 1980s. Taxol showed significant activity in human tumors in Phase I and Phase II clinical trials, especially in ovarian and breast carcinomas (McGuire *et al.* 1989; Holmes *et al.* 1991). The scientific community took a special interest in taxol at that time due to the lack of adequate treatment options available for ovarian cancer.

This knowledge established taxol as an important antitumor drug and stimulated a renewed interest in it. Intensive worldwide studies have reached explosive proportions since 1994 concerning its production, chemistry, biochemistry and many other aspects.

At that point, two problems needed solution before taxol could become a viable alternative as a useful treatment against any type of cancer. First, the lipophilic nature of taxol made it difficult to develop an acceptable dosage form for this drug. Gradually, this was overcome by the introduction of a suitable, "relatively" non-toxic dosage form.

Interestingly, the poor solubility characteristics of taxol might prove to be responsible for new discoveries regarding a problem of cross resistance to different classes of chemotherapeutic agents, caused by non-specific drug efflux and referred to as the MDR phenotype. The MDR phenotype is a gene that has been linked to multiple drug resistance (hence MDR); and some studies indicate that the solvent used for the delivery of taxol might have good activity against this common cause for therapeutic failure in the treatment of cancer (Woodcock *et al.* 1990; Webster *et al.* 1993; Fjällskog *et al.* 1993). It is known that this effect can result from the expression of plasma-membrane transport proteins (P-glycoproteins) which can enhance the efflux of structurally unrelated compounds from the cancer cell. At least three reports suggest that the solvent Cremaphore LH might enhance the antitumor actions of taxol when the tumor(s) display the MDR phenotype, and further work with cremaphores alone and in combination with other antitumor agents is needed to clarify this seemingly serendipitous finding.

Cremaphore LH is a form of ethoxylated castor oil and is responsible for many adverse drug reactions during the administration of taxol, and pretreatment with corticosteroids and antihistamines if often required to prevent allergic response up to and including anaphylaxis and death. Perhaps more difficult than this solubility concern was the procurement of an adequate supply of taxol for clinical trials and the anticipated needs for subsequent worldwide clinical use. Reported yields of taxol from the dried bark of *T. brevifolia* were averaging around 0.01%.

A large-scale process for the isolation of taxol was developed by Polysciences, Inc. (Paul Valley Industrial Park, Warrington, PA 18976); with yields of 0.005-0.01% (Boettner *et al.* 1979). Under these conditions, one kilogram of the bark could be expected to provide only 50-100 mg of taxol at best (or 30,000 lbs. being required for obtaining one Kg. of taxol). Approximately 2 grams of taxol are needed for one complete

course of therapy for a patient and this translates into requiring the bark from five to ten trees based on such reported yields.

Only a few studies on the taxol content of other species of *Taxus* were published from the time its importance was recognized in 1980 until 1992. From the bark of *T. wallichiana* were isolated taxol and the closely related cephalomannine, as well as other taxoids (Miller *et al.* 1981; Miller, 1980). From the bark of *T. baccata* L., Senilh *et al.* isolated nearly 20 different taxoids, including taxol, cephalomannine and a series of xyloside derivatives of these (Senilh *et al.* 1984). The taxoid content of the needles of *Taxus baccata* was studied and 10-deacetyl baccatin III isolated in relatively high yields (Chauviere *et al.* 1981). The fractions from the large scale (Polysciences) process from the bark of *T. brevifolia* were also investigated to recover any other taxoids with useful activity, or with possible semisynthetic utility such as conversion to taxol. However, only minute yields of 10-deacetylbaccatin III, 7-epitaxol and 10-deacetyl-10-oxotaxol were reported in these studies (Huang *et al.* 1986; Kingston *et al.* 1982); leaving a strong impression that the bark of *T. brevifolia* is a poor source for not only taxol, but also for any other useful analogues of taxol.

In spite of these problems, the bark of *T. brevifolia* has been accepted as the primary source for taxol until recently. However, since at the expected demand for taxol and the yields that can be realized from the bark, the yew tree population would be depleted in a few years, the use of the bark must stop. Among the alternatives that were being, considered to avoid this prospect are the following: 1) the use of needles, which are a renewable source, 2) growing the plant in tissue culture, 3) semi-synthesis from appropriate naturally occurring taxoids and 4) total synthesis. Progress has been made on all of these fronts.

As far as the needles are concerned, the most important candidate selected for direct isolation of taxol is the ornamental yew (*Taxus* x *media* cultivar Hicksii). Other

than analytical HPLC studies on the taxol content of the needles under various conditions, no practical methodology for the isolation of taxol or other taxoids has been published. Publications from this laboratory which address these issues are essentially the only work available in the literature (Rao *et al.* 1995; Rao *et al.* 1996). In addition to direct isolation of taxol, the needles were also examined for the presence of analogues such as 10-deacetyl baccatin III, since semi-synthesis from such is already an important alternative. The two most important species, *T. baccata* L. and *T. wallichiana* Zucc., have become the focus of attention since they were demonstrated to contain the highest concentrations of 10-deacetyl baccatin III.

Growing of various tissues of *T. brevifolia* in plant cell culture has been under development since 1990 and the methods have been standardized in many laboratories. However, the yields, as yet, have not been very attractive. Further research is expected to overcome this problem. Work on this alternative will continue due to the attractiveness of this approach and its potential for large-scale operations.

Starting with 10-deacetyl baccatin III, considerable progress was made in the area of semi-synthesis. In the first recorded semi-synthesis of taxol, the 13-cinnamate ester of 7-protected baccatin III was converted to the phenyl isoserine ester through a Sharpless hydroxy-amination (Denis *et al.* 1988). At this point, as an alternative to benzoylation of the amino group that will yield taxol, a t-BOC group (tert-butoxycarbonyl) was introduced, along with leaving the 10-hydroxyl free, to obtain an analogue known as taxotere. On the basis of its activity, taxotere has also been approved as an antitumor drug. Two important schemes for preparing taxol from 10-deacetyl baccatin III have been well developed and used for the large scale semi-synthesis of taxol as discussed in Chapter 2 (Denis *et al.* 1994; Ojima *et al.* 1991; Holton *et al.* 1992).

Two total syntheses of taxol have been recorded, and several other approaches towards the synthesis have also been reported in the literature as discussed briefly in Chapter 2 (Nicolaou *et al.* 1994a, 1994b, 1995a, 1995b, 1995c; Holton *et al.* 1994a, 1994b). Although these methods demonstrate remarkable achievements in the field of synthetic organic chemistry, they do not offer a practical method for the large-scale production of taxol or its analogues at this time.

Background of Research at the University of Florida

As part of one of these alternative quests, the National Cancer Institute hoped that instead of using the bark of the Pacific yew, the plant should be grown under hydroponic conditions, and they wanted to know whether plants grown in this manner would produce enough taxol for isolation. This laboratory was approached with this idea in early 1990, and with collaboration from Prof. George Hochmuth, Jr., of IFAS, University of Florida, the project was started. More on this aspect will be discussed in Chapter 6. In order to learn the current knowledge concerning the analysis and isolation of taxol, the pertinent literature was consulted. This yielded only a few papers on the isolation of taxol and taxoids, which were outlined above.

Methods

In general, the methods were found to be too cumbersome for others to repeat. For example, one of these publications in which isolation of taxol and its analogues was described from *T. wallichiana*, used the following steps, starting with the concentrated ethanolic extract of the plant:

1. Partition between water and hexane
2. Extraction of the aqueous phase with chloroform
3. Silica gel chromatography on the chloroform extract
4. A second silica gel chromatography

5. Counter-current distribution
6. HPLC on the appropriate fractions
7. A second HPLC on the appropriate fractions

Similarly, the large-scale process developed for the isolation of taxol by Polysciences Inc. from the bark of *T. brevifolia* consisted of the following steps, again starting with the alcoholic extract concentrate (Boettner *et al.* 1979).

1. Solvent partition water and CH_2CL_2, concentration to a solid
2. Separation of the extract solid into soluble and insoluble fractions
3. Chromatography on the soluble fraction
4. Recovery of taxol and crystallization twice
5. Silica chromatography on the taxol/ cephalomannine mixture
6. Recovery and crystallization of taxol

Thus, it appeared that, although procurement of taxol was of top priority, and many alternative approaches were attempted for solving this problem, one alternative, which was not considered, was to study the existing isolation procedure itself to make it more efficient. Thus the approach pursued at the University of Florida during 1990-91 was to develop a simpler process for the isolation. Over the next few months, a new process was developed based on the use of a single reverse-phase chromatographic column, and consisting of the following steps, starting with the alcoholic extract concentrate (Rao, 1993).

1. Partition between water and chloroform, and concentration
2. Reverse phase column chromatography on the extract directly
3. Harvesting the crystals and recrystallization.

The total chloroform extract of the bark of *T. brevifolia*, was applied directly to the C18-bonded silica column in 25% acetonitrile in water (i.e., no separation into soluble and insoluble fractions); and the column developed with a step gradient (30-60% acetonitrile). The column fractions were let stand for 3-7 days, whereby taxol and seven

of its analogues crystallized out directly from the fractions. These are filtered and purified further by recrystallization, or subjected to a small column.

This process using reverse phase column chromatography, gave not only higher yields of taxol (0.02-0.04% vs. 0.01%) on a pilot plant scale, but also made possible the simultaneous isolation of a number of analogues which have not been obtained from this plant before. These included a 10-deacetyl baccatin III (0.02%); and a number of xyloside analogues, chief among which being the 10-deacetyltaxol-7-xyloside, which can now be isolated in yields of 0.1% or higher.

Based on the successful fractionation of the bark extract of *T. brevifolia*, this technique was then ready for application to the other extracts such as the needles and wood of *T. brevifolia,* and to the needles of two other species of *Taxus*. These applications which gave practical methodology for processing these various extracts, also yielded many interesting taxoid compounds and these experiments are all detailed in this dissertation.

Although this work was started during 1991 and much of the expected work was completed by late 1993, the world-wide interest in taxol research made a "quantum leap" at about this time, with a phenomenal increase in publications dealing with all aspects of taxol chemistry. Some of the compounds which were isolated for the first time in this laboratory, and whose structures were determined, were rediscovered by others and published. In spite of the enormous increase in the number of relevant publications, most of these publications described the isolation of the minimum possible amounts of the compounds, often as amorphous solids. Many determined their structures only through NMR spectral interpretation, with little or no other physical characterizations, elemental analyses, derivatizations or reactions. In at least a few examples, the assigned structures were found to be wrong and were subsequently corrected once or

even twice. In the present work, practical isolation methods were used to obtain gram quantities of many compounds, as crystalline solids, where possible.

The compounds are usually characterized by physical and spectral properties, providing elemental analyses, and carrying out derivatization such as acetylation, oxidation, etc. The structures were elucidated through chemical reactions as well as through spectral data. Thus, even though some of the final structures may have been published, the work described here contains experiments that have not been carried out by these authors.

Brief descriptions of the topics that appear in this dissertation are given below.

Chapter 2 gives a brief and selected summary of the pertinent literature on taxol and taxoids, covering the areas of isolation, elucidation of structures, semi-syntheses and total syntheses. Because the subject matter expanded enormously since 1993, the scope of the review is limited to material that is relevant to the subject matter of the dissertation.

Chapter 3 deals with the taxoid composition of the needles of *Taxus brevifolia*. It covers the application of the reverse phase column chromatography to the needle extract, isolation of the major taxoid, brevitaxane A (or brevifoliol), along with brevitaxane B, and taxol. It continues with the elucidation of the structure of brevitaxane A by various reactions, as well as by a detailed analysis of the NMR spectral evidence.

Chapter 4 discusses some unusual reactions of brevifoliol. Such reactions have not been reported with this or any other taxoid compounds. In each case, crystalline compounds were obtained and characterized by physical and spectral data.

Chapter 5 deals with fractionation of the extract of the needles of *Taxus* x *media* cv. Hicksii by reverse phase column chromatography and isolation of taxol and several other taxoids and their characterization. In spite of the fact that this species (ornamental yew) was declared as the preferred plant for the future isolation of taxol, no publications

describing a suitable scheme for isolation of taxol or any other taxoids have appeared so far, other than analytical hplc data on their taxol content.

Chapter 6 is similarly devoted to the fractionation of the extract of the needles of *Taxus floridana* Nutt. by reverse phase column chromatography. Isolation of taxol, 10-deacetyl baccatin III, baccatin VI and a new crystalline taxoid compound named taxiflorine, with its structural elucidation are described.

Chapter 7 deals with the isolation of several crystalline non-taxane compounds present in the extracts of the bark and needles of *Taxus brevifolia*. These were shown to include flavonoids, phenols, and other types of compounds.

CHAPTER 2
A SELECTED REVIEW OF THE LITERATURE ON *TAXUS*

An overview of the taxol story has been presented in Chapter 1. In this Chapter, a selected review of the literature will be presented on taxol as well as the other taxanes, which are included in this dissertation. Two comprehensive reviews have been published on the subject of taxol, one by Kingston *et al.* (1993) and one by Miller (1980).

The present review on the genus *Taxus* may be roughly divided into two parts: studies before and studies after the discovery of taxol.

Earlier Studies

The genus *Taxus* (N.O. Taxaceae) represents a group of plants (common name, yew) which grow mostly in temperate climates and can be found distributed throughout the world. They are generally slow-growing evergreen trees or shrubs with stiff linear leaves (or needles); and fruits which are small, fleshy and bright red. The common names of the plants are qualified by the place of its origin, as for example, Pacific or western yew (*T. brevifolia,* Nutt.); European or English yew (*T. baccata, Pilg.*); Canadian yew (*T. canadensis,* Willd.); Japanese yew (*T. cuspidata,* Sieb. et Zucc.); Chinese yew (*T. chinensis,* Pilg.); Himalayan yew (*T. wallichiana,* Zucc.); ornamental yew (*T. x media* "Hicksii", Rehd.) and Florida yew (*T. floridana,* Nutt.).

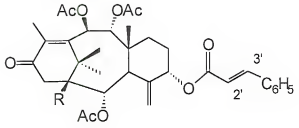
The toxic nature of this genus has been recognized for thousands of years, and in modern times, was first investigated chemically using the needles of *Taxus baccata* (Lucas, 1856). An amorphous mixture of alkaloids was isolated after extraction under acidic conditions and was given the name of "taxine." Further studies on taxine spanned

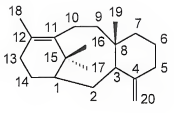
many decades and covered many reactions relevant to taxane chemistry. In one such study, Winterstein and Guyer (1923) were the first to show the presence of 3-dimethylamino-3-phenylpropanoic acid in the hydrolyzate of taxine and this acid later became known as "Winterstein's acid."

Until the 1960s most of the work on taxanes focused on these acid-extractable alkaloidal substances, which were readily separable from the large quantities of neutral, resinous materials which dominate the extract. Two groups of researchers were able to convert these somewhat unstable alkaloidal mixtures into more stable, non-basic substances in which the 3-dimethylamino-phenylpropanoic ester unit was transformed into a cinnamate ester. This, as well as the development of chromatographic techniques, made it possible to obtain pure compounds rather than mixtures.

Baxter *et al.* (1958) in England investigated the major cinnamate ester obtained from *T. baccata,* which they named 5-O-cinnamoyl taxicin-I triacetate [2-1]. Similarly, a Japanese team (Nakanishi & Kurono, 1963; Kurono *et al.* 1963) studied a cinnamate ester from *T. cuspidata*, and called it 5-O-cinnamoyl taxicin II triacetate [2-2] and the structures of both these can be seen in Figure 2-1. These two compounds differ only at C-1, where taxicin II lacks the tertiary hydroxyl found in taxicin I. The IUPAC numbering system for taxanes used throughout this dissertation can also be seen [2-3].

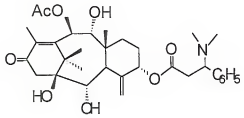
A few years earlier, Graf & Betholdt (1957) succeeded in isolating the purified basic alkaloids, taxine A and taxine B from the original taxine mixture. Taxine B was shown to have the structure [2-4] (see Figure 2-1); which corresponded with 5-O-cinnamoyl taxicin I triacetate, into which it could be converted via elimination of the dimethylamine moiety.



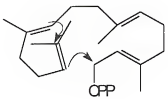


[2-1] - O-Cinnamoyl Taxicin I Triacetate, R = OH
[2-2] - O-Cinnamoyl Taxicin II Triacetate, R = H

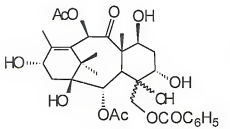
[2-3]-IUPAC Numbering



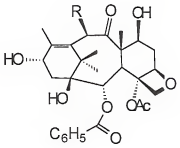
[2-4] - Taxine B



[2-5] - Geranylgeranyl pyrophosphate



[2-6a] - Proposed Glycol, later corrected to 6 b



[2-6b] - R = OAc, Baccatin II

[2-7] - R = OH 10-DAB-III

Figure 2-1 : Early Studies on the Constituents of some Taxus Species

Harrison (Harrison & Lythgoe 1966; Harrison *et al.* 1966) published one of the earliest biogenetic theories for the formation of taxanes starting with geranylgeranyl pyrophosphate and electrophilic cyclization [2-5]. Efforts by many groups to utilize a similar scheme to synthesize the taxane skeleton have been unsuccessful thus far (Kumagai *et al.* 1981; Hitchcock & Pattenden, 1992). Biogenetic pathways often provide ideas for simplified approaches in the synthesis of natural products.

Many early studies utilized acidic conditions for the extraction which might have hampered the isolation of neutral or acid-labile compounds, Kondo & Takahishi (1925) obtained a non-basic compound from the Japanese yew by using neutral conditions. The cinnamates can also be directly isolated from the plant, indicating that they occur naturally and also as artifacts of processing.

The National Cancer Institute (NCI) and the U.S. Department of Agriculture (USDA) joined forces in 1960 to collect and screen plants for activity in several animal tumor models. Arthur Barclay of the USDA collection team obtained samples from the Pacific yew tree (*Taxus brevifolia* Nutt., family Taxaceae) from Washington State in 1962. In 1964, the extracts from the bark and stems were found to be active against KB cells in vitro (Wani et al. 1971).

Studies after the Discovery of Taxol

Dr. Monroe Wall had discovered another antitumor agent known as camptothecin using the activity on KB cells for isolation and was interested in any other extracts showing this activity. Thus, work on *T. brevifolia* by Wani and Wall at the Research Triangle Institute was started and led to the isolation of 500 mg of taxol 2 years later in 1966. Cytotoxic actions (Wani *et al.* 1971) in KB cells, P388 leukemia, Walker 256 carcinosarcoma and P-1534 leukemia were present in the extracts from the bark. These

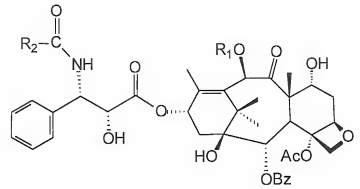
assays were all used at various points to monitor the fractionation, resulting in the isolation of taxol as the active principle.

In the 1960s the most straightforward and reliable method for the determination of complex chemical structures was x-ray diffraction analysis of a suitable crystal, also known as crystallography. Taxol crystallizes as thin needles not suitable for x-ray studies, but a tetraol derivative was amenable to x-ray studies. The structure of taxol [2-8] was determined by methanolysis (Figure 2-2); which yielded two compounds: the methyl ester of N-benzoyl phenylisoserine and an alcohol component shown to be a taxane tetraol [2-10a]. This tetraol skeleton was converted into a 7,10-bis-iodoacetate derivative and, unlike all of the taxanes studied earlier, taxol showed the following unique features:

1. A taxane skeleton with an oxetane ring system involving C-4, C-5 & C-20
2. An ester side chain consisting of N-benzoyl phenylisoserine at C-13
3. A carbonyl function at C-9

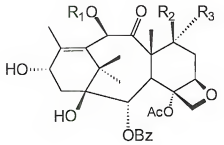
Baccatin III (Figure 2-1) and its 7α -epimer, baccatin V (Figure 2-2); were shown to be similar to taxol, having the oxetane ring and the C-9 carbonyl function. These epimers yielded better crystals and x-ray crystallography was performed. Baccatin III [2-6b] lacked the ester side chain present in taxol. Still another analogue, known as 10-deacetylbaccatin III (10-DAB, [2-7]) was later found to be much more widely distributed in *Taxus* spp., especially in the needles of *Taxus baccata*. This became an important taxane because it could be converted into baccatin III and later to taxol by the reattachment of the N-benzoyl phenylisoserine side chain at C-13 (See Figure 2-2).

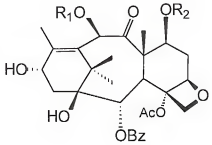
Taxol showed significant antitumor activity against a variety of *in vivo* murine tumors including B-16 melanoma and several human xenografts, which qualified it for



[2-8] - Taxol, R_1 = Ac R_2 = C_6H_5

[2-9] - Taxotere, R_1= H R_2 = $OC(CH_3)_3$



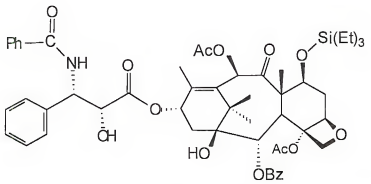


[2-10a] - Tetraol, R_1 = R_3 = H R_2 = OH

[2-10b] - Baccatin V, R_1 = Ac
R_2 = H, R_3 = OH

[2-11] - 7-O-TES 10-DAB III, R_1 = H
R_2 = Triethyl Silyl (TES)

[2-12] - 7-O-TES Baccatin III, R_1 = Ac
R_2 = Triethyl Silyl (TES)



[2-13] - 7-O-TES-13-O-Cinnamoyl Baccatin III

Figure 2-2 : Taxol and some Synthetic Targets

clinical trials. A few studies on other species of *Taxus* have also been published, which are referred to in Chapter 1.

Semi-synthesis of Taxol

The relative ease in ester formation of the three hydroxyl groups in 10-deacetyl baccatin III (10-DAB, [2-7]) are 7>10>>13. Esterification of the C-13 hydroxyl is very challenging due to the "inverted cup"-like folding of the taxane skeleton and strong hydrogen bonding with the carbonyl oxygen on the C-4 acetate. Before the side chain can be attached at C-13, the 7-hydroxyl must first be protected, often accomplished by attachment of a triethylsilyl group to give [2-11]. Next, this compound is acetylated at the 10-position, to form 7-triethylsilyl baccatin III [2-12].

In one method, [2-12] was esterified with cinnamic acid to give [2-13], which was then converted to the phenyl isoserine ester by the Sharpless hydroxyamination procedure (Sharpless *et al.* 1991) using osmium tetroxide and t-butyl-N-chloro-N-sodio-carbamate (Mangatal *et al.* 1989). The four isomers were separated and after deprotection of the hydroxycarbamates, N-benzoylation and deprotection of the 7-hydroxyl, taxol could be obtained.

During the investigations of Greene and Potier (Denis *et al.* 1988; Kanazawa *et al.* 1994) dozens of side chain analogues were synthesized and tested, resulting in the discovery of the taxol analogue known as taxotere [2-9]. Taxotere® (docetaxel) was found to be more active than taxol in the tubulin assay and animal tumor systems and has also been approved as an antitumor agent. In an alternative synthesis, the 7-protected baccatin III [2-12] was esterified using either the chiral β-lactam [2-14] or the oxazinone [2-15] derivative to yield taxol. This method or some variation is currently used for the semi-synthesis of taxol and taxotere commercially from 10-deacetyl baccatin III (Ojima *et al.* 1991, 1992).

Total Synthesis

Swindell (1992) published a review on the progress of more than thirty groups and reported "only modest success" in the total synthesis of taxol. Only two years later two separate groups headed by K. C. Nicolaou (Nicolaou *et al.* 1994b) at the Scripps Research Institute and R. A. Holton (Holton *et al.* 1994b) at Florida State University would announce almost simultaneously two total syntheses of taxol.

Nicolaou and colleagues designed the strategy for their synthesis based on the one bond disconnection analysis seen in Figure 2-3. After preparation of the fully functionalized A ring [2-16] and C ring [2-17] equivalents, a convergent and flexible

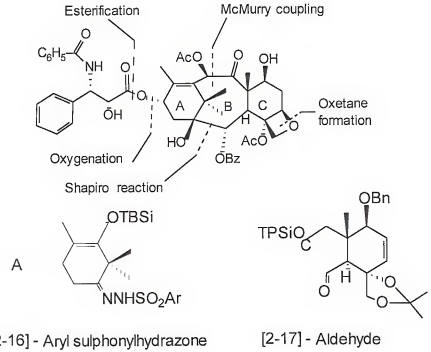


Figure 2-3 : Nicolaou's Retrosynthetic Strategy

synthesis of taxol involving 28 more steps allowed the preparation of numerous analogues. While not practical for the commercial production of taxol, synthetic methods

provide researchers with a source of analogues for structure-activity relationships and lead to better methods of production in general.

The first carbon-carbon bond between rings A and C was formed using a vinyllithium carbanion generated from the reaction of aryl sulphonylhydrazone [2-16], with *n*-butyl-lithium in tetrahydrofuran (THF); which was then combined with the aldehyde [2-17] in the Shapiro reaction (Shapiro, 1976) to produce [2-18] (Figure 2-3).

Regioselective epoxidation of the $\Delta^{1,14}$-double bond was completed in 87% yield with *t*-butyl peroxide in the presence of $VO(acac)_2$ leading to epoxide [2-19], which was then regioselectively opened with $LiAlH_4$ to give the 1,2-diol [2-20] with a 76% yield. The carbonate introduced between the C-1 and C-2 hydroxyls in the next step served to position the two rings for ring closure and also allowed for the stereo-controlled introduction of the 2α-benzoate later in the sequence. The dialdehyde [2-21] needed for cyclization of the B ring was obtained after standard deprotection of the two primary hydroxyls and mild oxidation with tetra-*n*-propylammonium perruthenate (TPAP) and 4-methylmorpholine N-oxide (NMO) in acetonitrile. The previous three steps provided the carbonate dialdehyde in 32% overall yield.

Formation of the B ring was accomplished with the versatile McMurry coupling (McMurry, 1989) under dilute conditions utilizing low valence titanium produced *in situ* from $(TiCl_3)_2$-$(DME)_3$ (10 eq.) and Zn-Cu (20 eq.) in 1,2-dimethoxyethane (DME) at 70 ^{o} C for 1 hour, giving the tricyclic A/B/C diol [2-22] with a 23% yield.

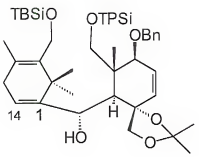
Selective acetylation of the hydroxyl at C-10 rather than C-9 was expected due to allylic activation and proceeded with 95% yield. Mild oxidation of the C-10 hydroxyl was then carried out with TPAP-NMO in acetonitrile analogous to the oxidation to the dialdehyde with a 93% yield.

After removal of the acetonide and protection of the primary hydroxyl at C-20 to make, the benzyl group was removed with catalytic hydrogenation and the 7-O-triethyl silyl protecting group was introduced to give [2-23]. Selective deacetylation of the primary acetate then provided the triol for the formation of the oxetane of ring D, which involves monotosylation at C-20 (primary OH) and triflate formation at C-5 (secondary OH) to produce [2-24]. Oxetane formation with a 60% yield occurs after mild acid treatment with catalytic camphorsulfonic acid (CSA) in methanol, followed by treatment with silica gel in dichloromethane.

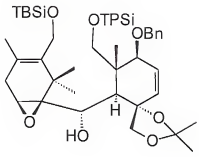
Acetylation of the C-4 position (tertiary hydroxyl) was followed by regioselective ring opening of the carbonate to the hydroxybenzoate functionality, both with good yields. The C-13α oxygen is introduced with pyridine chlorochromate in 75% yield followed by stereospecific reduction of the ketone [2-25] using $NaBH_4$ in methanol in excess, for 83% yield. The hydroxyl is esterified using Ojima's β lactam synthon [2-14] (Figure 2-2) using the strong base sodium-hexamethyldisilazane for 87% yield based on 90% conversion. Removal of the triethylsilyl groups with hydrogen fluoride in pyridine (HF-Pyr) completes the synthesis of taxol in 80% yield.

Other Synthetic Approaches

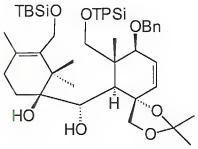
As previously mentioned Holton's group published a total synthesis of taxol in early 1994 at about the same time as Nicolaou, but their approach was quite different, with only a few reactions in common. Studies involving the fragmentation of bicyclic epoxy alcohols, referred to as "epoxy alcohol fragmentation," were the cornerstone of their syntheses of bicyclo[5.3.1] systems, including the unnatural epimer of (+)-taxusin [2-26], known as (-)-taxusin or *ent*-taxusin [2-27] (Figure 2-6).



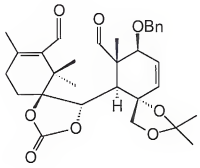
[2-18]



[2-19]

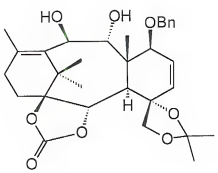


[2-20]

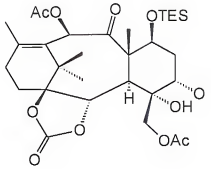


[2-21]

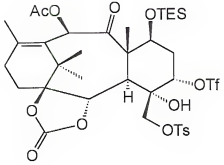
Figure 2-4 : Nicolaou's Taxane Ring Synthesis



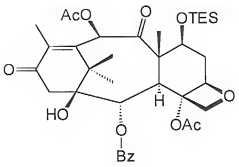
[2-22]



[2-23]

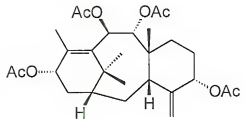


[2-24]

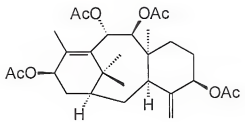


[2-25]

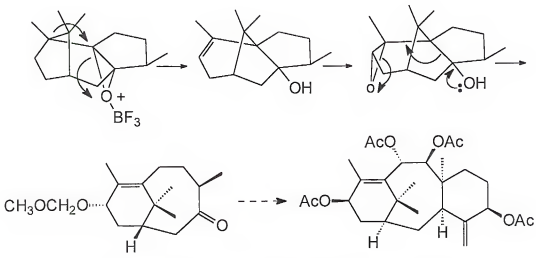
Figure 2-5 : Nicolaou's Final Synthetic Intermediates



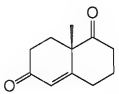
[2-26] - natural (+)-Taxusin



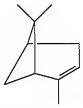
[2-27] - *ent*-(−)-Taxusin



Holton group - Patchouline Oxide Fragmentation



[2-28] - Wieland-Miescher ketone
Danishefsky group



[2-29] - α-Pinene
Wender group

Figure 2-6 : Starting Points of Other Synthetic Strategies

Danishefsky's total synthesis of baccatin III in 1996 (and hence, taxol); borrowed extensively from the experiences of Ojima, Holton, Nicolaou and others. The Weiland-Miescher ketone [2-28], available through catalytic asymmetric induction, allowed the installation of all stereochemical requirements to reach baccatin III in a sequential fashion. According to Danishefsky, "Our synthesis, though arduous, involves no relays, no resolutions, and no recourse to awkwardly available antipodes of the chiral pool" (Danishefsky *et al.* 1996).

Wender's group published a most concise synthesis involving α–pinene [2-29] for construction of the ABC-tricyclic core of the taxanes (Wender & Rawlins 1992). Their approach takes advantage of the tendency for C-7 to undergo facile aldol/reverse aldol epimerization in taxol, allowing for aldol condensation under very mild conditions.

General Structural Features of Taxanes

The taxanes comprise a relatively large group of diterpenoid natural products covering a variety of structural patterns. These are believed to arise from geranyl geraniol [2-16], although the exact biosynthetic route has not been completely elucidated. A brief discussion of the major structural variations of taxanes is relevant to this work because many of these structures have been found in the compounds isolated in this work. A number of different forms that the C-20 diterpene skeleton itself can assume have been isolated. Next, the oxidation states, esterification patterns of the hydroxyls, and presence or absence of basic or neutral side chains allow for the extensive structural variation seen in these compounds.

The taxane skeleton is a specific diterpene structure, consists of 20 carbon atoms arranged in a fused tricyclic system with the 6, 8 and 6 members in rings A, B and C, respectively. The double bonds at 11/12 and 4/20 are part of the basic ring system, although the latter may be modified by oxygenation to an epoxide or more commonly to

an oxetane. As in the case of the analogous steroids with the two methyl groups as part of the skeleton, the taxanes have four methyl groups #16, 17, 18 and 19 as part of the taxane ring system. Some examples of the taxane skeleton found in the various species of *Taxus* are shown in Figures 2-1 and 2-2.

Oxygenation of the taxane ring has been observed to varying extents. The minimum number being 4, distributed at 5, 9, 10 and 13, as seen in taxusin [2-26]. In general, oxygenation may occur at carbons 1, 2, 4, 5, 7, 9, 10 and 13. Instances have been recorded where oxygenation was present at 14 (in place of 13), as well as part of the methyl groups at 19 and 17.

Taxa-4(20);11-dienes

This is the most common structural type seen in the taxanes, with a C-4(20) and a Δ^{11} double bond. These taxanes are generally referred to taxa-4(20);11-dienes. The alkaloidal Winterstein esters are included in this group as are many of the neutral taxanes. The oxygen at C-9, if present, is usually seen as a secondary alcohol or as an ester. The C-13 position in this group, likewise, exists as an alcohol, ester or oxidized to a carbonyl to form an α,β-unsaturated carbonyl. Esterification at C-13 is usually limited to an acetyl or a cinnamoyl, but the side chain (N-acyl phenyl isoserine); as found in taxol, cephalomannine and others has not been reported in this subgroup so far. The 5 position is oxygenated with an α-hydroxyl, which might be free, or esterified by an acetic acid, cinnamic acid or the Winterstein-acid. Some examples of these compounds with a cinnamate ester function are described in Chapter 5.

4(20)-Epoxides

This group is relatively less frequent but examples with different substitution patterns have been isolated. One variation comes from the presence or absence of hydroxyl at C-1. Members of this subgroup also generally contain the 5-α-hydroxyl,

which is esterified in the same fashion as the dienes above to provide further variation. An unusual example is the taxane with the C-9-nicotinoyl ester function, found in *Austrotaxus spicata* Compton Taxaceae (Ettouati *et al.* 1988).

Oxetanes

This group is characterized by having an oxetane ring system involving the carbons 4, 5 and 20. It may be divided into two subgroups based on whether they contain the phenyl isoserine ester side-chain at C-13 or not. The former contains taxol and all of the other compounds, which are active in the tubulin assay and hence are of much importance. Division into two other subgroups is also possible in those without the C-13 side chain, with one having a carbonyl at C-9 and with a hydroxyl or an esterified hydroxyl at C-9.

The oxetane-containing taxanes are generally highly oxygenated and often have oxygen at C-1, 2, 4, 5, 7, 9, 10, and 13. In some special instances, a hydroxyl has been reported at C-19 (Fuji *et al.* 1993). The phenylisoserine ester side chain has been seen in the form of at least three different amides that occur in nature. These are taxol, with the N-benzoyl group, cephalomannine, with the N-tiglioyl group and taxol C, with the N-hexanoyl group.

Taxol has a complex structure and knowing what features of this structure are necessary for the activity is of utmost importance and this aspect has been studied using the *in vitro* tubulin binding, and the cell culture assays and a summary of these data is presented below (Samaranayake *et al.* 1993).

Acylation of the 2' position of taxol does not destroy cytotoxicity but does stop promotion of microtubule assembly. Bulky acyl groups reduce the activity in the cell culture, thus suggesting that hydrolysis of the 2' position back to a free hydroxyl might be required.

Substitution of the 7 position does not appear to significantly decrease the activity. Taxanes with a 7β-O-xyloside moiety are comparably active in both assays when compared to the respective aglycones. Similarly, epimerization at the 7-position does not eliminate activity.

Hydrolysis of the 10-acyl function does not reduce the cytotoxicity significantly in cell culture assays. As with other structural features, this point is being explored in the more recent clinical trials in Europe with taxotere.

The importance of the oxetane ring for activity has been investigated through ring opening via different Lewis acids including Meerwein's reagent (triethyloxonium tetrafluoroborate); acetyl chloride, mesyl chloride and others. The product obtained form the Meerwein's reagent had a primary alcohol at C-20 and secondary C-5-hydroxyl, but no other changes compared to taxol. The activity normally seen with taxol in both assays was lost with the opening of the oxetane ring. This suggests that the oxetane ring is necessary for activity but leaves open questions regarding the effect of ring contractions in ring A.

The properties of the C-13 hydroxyl mentioned above make attachment of a side chain quite difficult. Protection of other free hydroxyls in both the side chain and taxane skeleton are necessary, followed by selective deprotection after the side chain has been attached. Taxotere and taxol have both been synthesized from this taxane and this is currently the starting material for the production of both drugs.

Epimerization of the 7 hydroxyl from β to α *via* a retro-aldol mechanism allows formation of an energetically favorable hydrogen bond with the 4-acetate carbonyl oxygen. This epimerization is a concern in both taxane isolation and synthetic methods, and necessitates the avoidance of acidic or basic conditions. Protection of this C-7 β-

hydroxyl with groups such as a chloroacetate avoids both epimerization and unwanted reaction at this position.

Abeotaxanes

A number of taxanes in which the A-ring is isomerized to a 5-membered ring to give a 5/7/6 instead of the 6/8/6 system have been isolated and these are termed abeotaxanes. They are again divided into two groups into a) those with the 4/20 unsaturation and b) those with an oxetane ring at this location. We isolated the first members of each of these groups in our work, e.g. brevifoliol (Chapter 3); and the compounds isolated from the bark of *T brevifolia* described in Chapter 6. As indicated earlier, treatment of taxol with acidic reagents can isomerize ring A to form such compounds, although these compounds are naturally present in the extract and not artifacts.

CHAPTER 3
TAXANE CONSTITUENTS OF THE NEEDLES OF *TAXUS BREVIFOLIA*

Taxol was originally isolated from the bark of the Pacific yew (*Taxus brevifolia* Nutt., N.O. Taxaceae). As indicated in Chapter 1, during 1991-1993 there was a reassessment of the use of the bark as the source. This concern resulted in an intense search for alternative sources for taxol that are renewable, with sources such as the needles of the yew tree instead of the bark. This laboratory was also involved in this search and looked into the needles of three different yew species as a source for taxol: *T. brevifolia*, *T.* x *media* Hicksii and *T. floridana*. The taxane composition of *T. brevifolia* needles is the subject of this chapter.

Fractionation of the Needles of Taxus brevifolia

A quantity of 100 lbs. of the needles of *T. brevifolia* was obtained from a supplier in Oregon. They were air-dried and extracted with methanol at room temperature and the extract was concentrated under reduced pressure to a syrup. This was partitioned between water and chloroform, and the organic layer concentrated to give a dark greenish brown semi-solid, called "extract solids", which represented about 5% of the dry weight of the needles.

It was decided to follow the method successfully developed with the bark extract for the fractionation of the extract solids, using preparative scale, reverse phase column chromatography. Direct application of the crude chloroform extract of the needles onto a C-18 bonded reverse phase silica column was accomplished as described in the experimental section. After placing the extract-containing silica onto a 25% acetonitrile

in water column (1:4 ratio of loaded to clean silica); a step gradient of acetonitrile in water mixtures was performed up to 60% acetonitrile.

Preliminary studies on the extract solids of the needles by TLC and analytical HPLC showed that the sample contained somewhat minor amounts of taxol. A predominant component that was slower moving than taxol in TLC gave a greenish-blue colored spot when sprayed with 1 N sulfuric acid and heated on a hot plate (charring). Likewise, in the analytical HPLC, this component appeared after 10-deacetyl baccatin III as the major constituent judging from the peak heights, but before taxol and at least several times more abundant.

The reverse phase column (C-18 bonded silica gel) on the needle extract concentrate was started with 25% acetonitrile in water. The sample was carefully prepared as a slurry (see experimental) and added to the column. The column was developed using a step gradient of acetonitrile in water 30-60%. Fractions of suitable volume were collected and monitored by absorbance at 275 nm, TLC and analytical HPLC. Four regions were recognized in the elution profile of the column, based on the UV absorbance (275 nm.); which contained the resolved constituents of the extract.

The early fractions contained components, which accounted for the bulk of the UV absorbance of the sample. These appeared to be non-taxane phenolic compounds with or without attached sugars. A description of these will be given in Chapter 7. The first taxane component, which appeared at the 35-40% acetonitrile elution, was also the major component. It was collected from the appropriate fractions, and after concentration, obtained as a crystalline solid. Next, fractions from the 50% acetonitrile elution contained taxol, which was obtained as a crystalline solid directly from the fractions. Following this, the fractions from the 55-60% acetonitrile elution gave another taxane component which gave a greenish blue spot on the TLC (after charring with sulfuric acid) similar to the major constituent referred to above.

Brevitaxane A (Brevifoliol) [3-1]

The major constituent, which was obtained in a yield of 0.2-0.25%, was named brevitaxane A because the physical and spectral data indicated that it was a new taxane compound (later renamed by others as brevifoliol, which will be used throughout this dissertation). Elemental and FAB-MS analysis (MH+ 557) agreed with the molecular formula of $C_{31}H_{40}O_9$ (Balza *et al.* 1991).

An examination of the ^1H NMR spectrum showed the presence of two acetyl groups (signals at δ 1.76 and δ 2.07); and a benzoate group {δ 7.88 (d); δ 7.43 (t) and δ 7.56 (t)}. The spectrum also gave evidence for the presence of a (4/20) exocyclic double bond (two characteristic broad singlets at δ 4.82 (H-20A) and δ 5.20 (H-20B) and signals at δ 112.1 (C-20) and δ 149.0 (C-4) in the ^{13}C NMR spectrum.

Very little information on the various types of taxane structures that are known now was available at that point in time (1991) and even less on their diagnostic spectral characteristics. Based on analogous taxanes and the evidence outlined above it was postulated that this major constituent had the relatively common 4/20,11-taxadiene type skeleton. The presence of an exocyclic 4/20 double bond and absence of an oxetane ring supported our initial assumptions. The next step was to determine the positions of the various substituents in the molecule in order to elucidate the complete structure.

Most of the structural elucidations of taxanes at the time were based on degradative studies. It was decided to follow this lead in establishing the presence of the various functionalities as well as their location in brevifoliol, by actual reactions and/or derivatizations, supplemented by spectral methods.

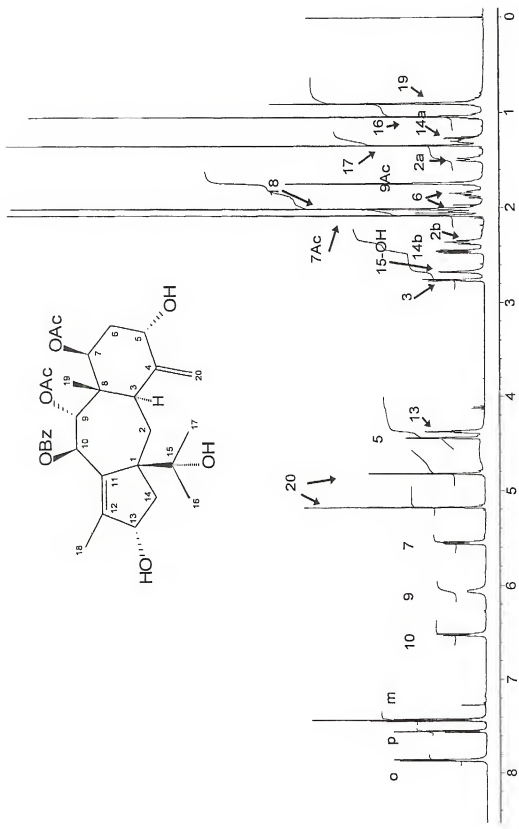


Figure 3-1 : Proton NMR Spectrum of Brevifoliol

Hydroxyl Functionalities

i) Acetylation: To determine the number and positions of all hydroxyls in the molecule, the compound was subjected to acetylation. Two products were obtained under mild conditions (20 ° C, 15 min). These two were separated by chromatography and both obtained as crystalline solids. One was shown to be a monoacetate and the other a diacetate.

Table 3-1 : Proton NMR Spectra of Brevifoliol and Brevifoliol Acetates

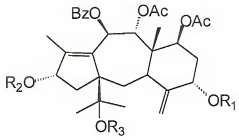
Position	Brevifoliol (J in Hz) [3-1]	Brevifoliol 5 – Ac [3-2]	Brevifoliol 13 – Ac [3-3]	Brevifoliol 5,13 – Ac [3-4]	Brevifoliol 5,13,15 – Ac [3-5}
2	1.49 cm	1.46 cm	1.47 cm	1.46 br d (13)	1.53 br d(13)
	2.36 dd (9,13)	2.40 dd(9, 13)	2.42	2.41 dd(9, 13)	2.65 dd(9,
3	2.78 d (9)	2.76 br d (9)	2.91 d (9)	2.72 br d (9)	2.71 br d (9)
5	4.45 br s	5.37	4.37 br s	5.39 br s	5.38 dd (4, 2)
6	1.86 cm	1.88 cm	1.85 cm	1.90 cm	1.87 cm
	2.02 cm	2.0 cm	1.99 cm	2.00 cm	2.0 cm
7	5.56 dd (5,11)	5.62 dd (5, 11)	5.66	5.61 dd (5,	5.63 dd(5,
9	6.05 br	6.03 br d(10.6)	6.07 d	6.09 br	5.8 d (10.8)
10	6.53 d (10.6)	6.63 d (10.6)	6.66 d	6.65 d (10.6)	6.64 d (10.8)
13	4.38 t (7.5)	4.53 br t (7.2)	5.46 br s	5.54 br t (7.2)	5.61 t (6.9)
14	1.29	1.22 dd *	1.32 cm	1.25 dd *	1.25 dd *
	2.46	2.42 dd *	2.51 cm	2.51 dd *	2.62 dd *
16	1.05 s	1.03 s	1.09 s	1.11 s	1.63 s
17	1.35 s	1.33 s	1.35 s	1.35 s	1.71 s
18	2.01 s	2.06 s	2.02 s	2.03 s	1.96 s
19	0.90 s	0.91 s	0.89 br s	0.92 s	0.92 s
20 A	4.82 br s	4.90 br s	4.80 br s	4.92 br s	4.89 br s
20 B	5.20 br s	5.28 br s	5.15 br s	5.28 br s	5.29 br s
o-Ph1	7.88 d (7.5)	7.87 d (7.5)	7.87 d (7.5)	7.87 d (7.5)	7.84 d (7.5)
m-Ph1	7.43 t (7.5)	7.43 t (7.5)	7.44 t (7.5)	7.44 t (7.5)	7.42 t (7.5)
p-Ph1	7.56 t (7.5)	7.55 t (7.5)	7.56 t (7.5)	7.56 t (7.5)	7.53 t (7.5)
-	1.76 s	1.76 s	1.76 s	1.75 s	1.77 s
__	2.07 s	2 06 s	2.05 s	2.02 s, 2.07 s	2.02 s, 2.08 s
		2.13 s	2.06 s	2.08 s	2.09 s, 2.11 s

NMR were recorded at 600 MHz in $CDCl_3$ on a Varian Unity 600 instrument at ambient temperature. Chemical shifts δ (ppm) are reported with TMS as internal standard.

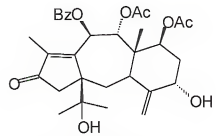
Table 3-2 : Carbon NMR Spectra of Brevifoliol and Brevifoliol Acetates

Carbon Number	[3-1] Brevifoliol	[3-2] 5-Ac	[3-3] 13-Ac	[3-4] 5,13-Di-Ac	[3-5] 1,5,13-Tri-Ac
1	62.4	63.0	63.4	63.0	63.3
2	29.1	29.2	29.4	29.1	28.3
3	37.9	38.8	37.6	38.8	38.9
4	149.0	145.4	147.4	145.2	145.1
5	72.4	74.1	72.7	74.1	73.9.
6	36.0	33.9	36.1	33.9	34.0
7	70.1	69.7	69.8	69.6	69.7
8	45.0	44.8	45.2	44.8	45.0
9	77.1	77.9	79.8	79.3	78.9
10	70.2	70.7	70.3	69.8	68.4
11	133.9	134.0	136.5	136.4	136.5
12	151.5	151.1	150.5	147.3	148.2
13	76.7	76.9	77.8	76.9	78.0
14	47.3	47.1	44.2	44.1	43.3
15	75.9	75.6	75.6	75.6	87.2
16	26.9	27.0	27.0	27.0	23.1
17	24.8	24.8	25.0	24.8	21.8
18	12.0	11.8	12.1	11.9	11.9
19	12.9	12.9	12.9	12.9	13.5
20	112.0	114.1	111.5	114.3	114.3
CO-C_6H_5	164.3	164.1	164.2	164.1	165.0
Bz-ipso	129.3	129.2	129.4	129.1	129.9
Bz-ortho	129.4	129.4	129.5	129.5	129.3
Bz-meta	128.7	128.7	128.7	128.8	128.4
Bz-para	133.2	133.3	133.2	133.4	133.0
CO-CH_3	20.7 21.4	20.8 21.4 21.2	20.7 21.4 21.1	20.7 21.4 21.2 21.0	20.8 21.4 21.3 21.0 21.7
$COCH_3$	169.9 170.5	169.9 (X2) 170.2	169.9 170.8 169.7	169.91 170.5 169.6 169.9	169.9 170.5 169.6 171.0 169.5

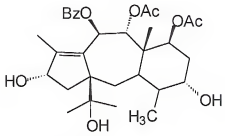
^{13}C NMR spectra were recorded at 150 MHz in $CDCl_3$ on a Varian Unity 600 spectrometer at ambient temperature. Chemical shifts δ (ppm) are reported with TMS as internal standard.



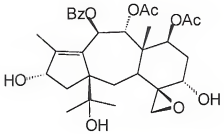
	R_1	R_2	R_3
[3-1] -	H	H	H
[3-2] -	Ac	H	H
[3-3] -	H	Ac	H
[3-4] -	Ac	Ac	H
[3-5] -	Ac	Ac	Ac



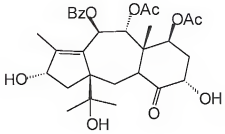
[3-6] - 13-Ketone



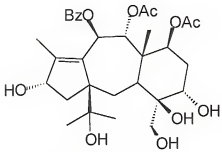
[3-7] - 4,20-Dihydro



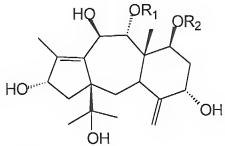
[3-8] - 4,20-Epoxide



[3-9] - Norketone



[3-10] - 4,20-Diol



[3-11] - Hydrolysate, $R_1 = R_2 = H$
[3-15] - Debenzoyl, $R_1 = R_2 = Ac$

Figure 3-2 : Brevifoliol and Reaction Products

Appropriate conditions under which each of these could be obtained as exclusive products were developed. At room temperature in acetic anhydride for 1-2 minutes before quenching the reaction, the monoacetate was the major product (>90%). Likewise, at 80 ° C for 30 min. the product was the diacetate.

The ^{1}H NMR spectral data for the monoacetate showed that the signal at δ 4.45 (br s) shifted to δ 5.37 (dd, J=4.2, 2.4 Hz); indicating that acetylation took place at the 5-OH, as shown in [3-2]. In the diacetate, besides this shift for the 5-OAc, the signal at δ 4.38 (t, 7.5 Hz) shifted to δ 5.54 (br t, 7.2 Hz); thus showing that the second acetate was located at C-13 [3-4]. A naturally occurring brevifoliol 13-acetate [3-3] was isolated and 1,5,13-brevifoliol triacetate [3-5] produced in this lab will be discussed in Chapter 4.

ii) Oxidation: Brevifoliol was readily oxidized by manganese dioxide (MnO_2) in refluxing benzene to yield a ketone product. In the ^{1}H NMR spectrum, a major change was the absence of the triplet at δ 4.38 due to the C-13 proton, thus showing that the oxidation took place at the 13-OH [3-6]. Further evidence was seen by the shift of the signals for the C-14 protons from their normal positions at δ 1.29 (dd, 14.0, 7.6 Hz) and δ 2.46 (dd, 14.0, 7.6 Hz) to δ 2.32 (d, 19 Hz, H-14α) and δ 2.48 (d, 19 Hz, H-14β). When brevifoliol was oxidized by Jones reagent, the same 13-keto brevifoliol seen with MnO_2 initially formed [3-6]. With time the initial product gradually disappeared, giving rise to a faster moving product. This second oxidation product was shown to be the result of an unusual reaction described in Chapter 4.

4/20 Unsaturation

i) Hydrogenation: When hydrogenated in the presence of 5% Pd/carbon, brevifoliol gave the dihydro derivative [3-7]. In its ^{1}H NMR spectrum, the characteristic signals at δ 4.82 and δ 5.20 due to the C-20 protons were absent and a new methyl

doublet and a new methine proton appeared. In the ^{13}C NMR spectrum the characteristic signals from the exocyclic 4/20 double bond were absent, accompanied by the appearance of new methyl and methine signals.

ii) Epoxidation: Brevifoliol was heated in dichloromethane with meta-chloro peroxybenzoic acid (MCPBA); whereby it underwent oxidation to yield the epoxide [3-8], a crystalline compound.

iii) Ozonization: Brevifoliol has two double bonds, one at the 11/12 position and the other at the 4/20 position. Of these, the former is tetra-substituted, while the latter is of an exocyclic methylene type. No information was available in the literature regarding the reactivity of the taxane skeleton to indicate whether one or both double bonds would be cleaved by ozonolysis. In the present work, ozonization was carried out in a mixture of methanol and dichloromethane –70 ° C. After the disappearance of the starting material, the ozonide was decomposed with dimethyl sulfide and the products isolated by chromatography. Two major products were separated. The first was the same as the epoxide [3-8] obtained by reaction with MCPBA. The second was the expected ozonolysis product in which the 4/20 double bond was cleaved to form the ketone [3-9].

iv) Formation of a diol: As one of the characteristic reactions of an ethylenic function, oxidation by osmium tetroxide was attempted with brevifoliol. The reaction proceeded smoothly to give a diol [3-10].

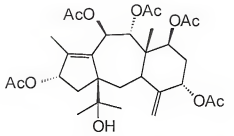
Number and Nature of the Oxygen Substitution

From the preceding discussion it is evident that brevifoliol has two free hydroxyls, two acetoxyls and one benzoyloxy functions. However, in the ^{13}C NMR spectrum of brevifoliol, the number of oxygen substituted carbons was six: δ 70.1, δ 70.2, δ 72.4, δ 75.9, δ 76.7 and δ 77.2. To determine if one of the six is a different type of an ester, or a tertiary hydroxyl, brevifoliol was subjected to saponification in alcoholic KOH to yield the

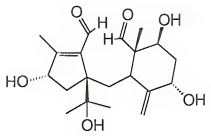
hexaol [3-11], obtained as a crystalline solid. This was then acetylated to a crystalline acetate [3-12]. The ^{1}H- and ^{13}C NMR spectra of [3-12] showed the presence of 5 acetates (^{1}H: δ 21.8, δ 21.7, δ 21.4, δ 21.3, δ 21.0, δ 20,8; and ^{13}C δ 171.0, δ 170.4, δ 169.8, δ 169.6 and δ 169.5); which suggested that brevifoliol contained a tertiary hydroxyl.

In the conventional taxane skeleton, a tertiary hydroxyl is often present at C-1, with the other hydroxyls (or esters) at C-2, C-5, C-7, C-9, C-10 and C-13. Thus, with brevifoliol having five oxygen substituents, one of these positions must be without attached oxygen. Thus, it would be important to know which of these positions does not have an oxygen substituent. For this reason, the hexaol [3-11] was subjected to oxidation by periodate. If there were two pairs of vicinal hydroxyls, e.g. 1,2 and 9,10, the hexaol will be cleaved in such a way as to give smaller molecules which represent the A and C rings. If there is only one such pair, the reaction will produce a product with all of its carbons intact. The hexaol underwent oxidation readily to form a dialdehyde [3-13] without losing any carbon atoms found in original carbon skeleton. Unaware of the unusual A ring structure, it was presumed that the presence of a tertiary hydroxyl at C-1 precluded the presence of oxygen substitution at C-2. Additional evidence for a methylene carbon at C-2 was found in the COSY spectrum from the chemical shifts in the H-3β-H-2α–H-2β isolated spin system.

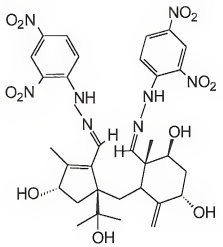
Thus, brevifoliol has two hydroxyls at 5 and 13. Locating the benzoate group at one of the three choices, 7, 9, or 10 will elucidate the structure. At this point, brevifoliol was required in microbial and fungal biotransformation project in our laboratory. In order to produce an antiseptic sample an aqueous alcoholic solution was sterilized in a steam autoclave at 125 ° C, 20 atm., to see if it is stable. It was found that the compound underwent degradation to give two or three products.



[3-12] - Pentaacetate from Hexaol



[3-13] - Periodate Oxidation



[3-14] - Periodate Oxidation Osazone Product

Figure 3-3 : Brevifoliol Hexaol Reaction Products

The major component of this mixture was found to be debenzoyl brevifoliol (Figure 3-1 [3-15]). Of the three possible locations, 7, 9, and 10 for the benzoate, only 10 is allylic and hence the ester at this position is more likely to be labile. Taxol with the benzoate at C-2 is completely stable to heat and pressure for hours. This evidence, along with chemical shift arguments concerning the effect of acetylation versus benzoylation, led us to place the benzoate at C-10.

This group presented the isolation and the structural elucidation of brevitaxane A at the International Research Congress on Natural Products held in Chicago, IL in July

1991. Balza *et al.* (1991) published the isolation of a new compound at about that same time from the needles of *T. brevifolia*, which they named brevifoliol, and an assignment of its structure as shown in [3-16]. The compound appeared to be similar to, if not the same as, brevitaxane A, that was isolated from the needles at the University of Florida. The structure proposed by Balza *et al.* differed from that of brevitaxane A, with the benzoate group being placed at C-7 instead of at C-10.

That same year (1992); the isolation of taxchinin A was described (Fuji *et al.* 1992); which was later shown to be 2-acetoxy-brevifoliol. Fuji correctly assigned the structure with a 5-membered ring A, on the basis of NMR spectral data. The authors who isolated brevifoliol and assigned structure with the 7-benzoate (Chu *et al.* 1993) published a revised structure for brevifoliol, in which the benzoate was moved to C-10, from C-7, but with the skeleton of a conventional taxane.

During 1993, two other publications appeared, one from Georg *et al.* (1993). and the other from Appendino *et al.* (1993) reexamining the NMR spectral data of brevifoliol, and arriving at the structure in which the A-ring was 5-membered. Later that year, Chu *et al.* (1993); on the basis of x-ray crystallographic data, revised the structure of brevifoliol again to the presently accepted structure.

Due to the intense competition in "taxol research", we began a detailed examination and analysis of the NMR spectral data using the ^{13}C NMR, NOESY, HETCOR and other spectral methods to determine if the rearranged (5/7/6) skeleton might be distinguishable from the spectrum of a taxane with a conventional (6/8/6) skeleton. The following is an analysis of the spectral data of brevifoliol.

The carbonyl signal in the ^{13}C NMR spectrum at δ 164.3 indicated the presence of one benzoate, and signals at δ 169.9, δ 170.5, likewise, indicated that 2 acetate ester groups were present. Further support for the benzoate was obtained by the four

aromatic signals between δ 128.7 and δ 133.2 (see tables 3-1and 3-2); and for the two acetates, by the methyl signals at δ 20.7 and δ 21.4. Analysis of the ^{1}H NMR and ^{1}H COSY and $^{1}H,^{13}C$ Heteronuclear Correlation (HETCOR) experiments also gave additional support for the presence of the acetates with signals at δ 1.76 s and δ 2.07 s, as well as benzoate signals at δ 7.88 d (ortho); δ 7.43 t (meta); and δ 7.56 t (para). Next, evidence for the presence of the normally present (11/12) taxane double bond could be seen in the carbon spectrum by the signals at δ 133.9 (C-11) and δ 151.5 (C-12);. Similarly, the existence of a (4/20) exocyclic double bond could be seen by the signal δ 149.0 (C-4) and δ 112.1 for (C-20). In the ^{1}H NMR spectrum the exocyclic 4/20 double bond is also indicated by the two characteristic broad singlets seen at δ 4.82 (H-20A) and δ 5.20 (H-20B).

In the ^{1}H COSY experiment weak but definite interactions between the singlet at δ 4.82 (C-20a) with both the H-3β doublet at δ 2.78 (9 Hz.) and the H-2α doublet of doublets at δ 2.36 (9, 13 Hz.) supported the assignments given for the methylene protons. (The designations for the C-20 protons are A and B, since α and β do not have the conventional meaning system and could be confusing). Along with the interaction between H-2α and H-2β the first isolated spin system in the ^{1}H spectrum was established and the relative geometry of the protons.

The region between δ 62.4 and δ 77.1 in the ^{13}C spectrum carbons with hydroxyl or ester oxygen attached to oxygens, and these signals could be further defined in the DEPT experiment (Distortionless Enhancement with Polarization Transfer, NMR) as primary, secondary, tertiary and quaternary carbons. The spectrum showed two quaternary carbon signals and five oxymethine carbon signals. Since the presence of only six signals was expected based on the proposed formula, the quaternary signal at δ 62.4 was intriguing even from the start of the spectral examination. In taxol with its C-9

carbonyl, the C-8 signal appears near δ 58, so the signal at δ 62.4 immediately raised questions about the true structure of brevifoliol. This signal did not fit the normal chemical shift pattern of any naturally occurring taxanes known at that time. In the absence of a carbonyl group at C-9, the C-8 carbon usually falls in the region of δ 40-50 ppm.

Unable to satisfactorily explain this unusual peak position, the Chemistry Department was contacted about crystallographic services. X-ray crystallographic analysis was performed by Dr. K. A. Abboud on the 5-monoacetate [3-2]. Surprisingly, the presence of an unusual 5/7/6 ring system was evident, where the normal 6-membered A ring of the conventional taxane system was "rearranged" to form a 5-membered ring with the carbons 15, 16 and 17 moved out of the ring system to form a hydroxy isopropyl group at C-1. Since the x-ray structure was obtained on brevifoliol-5-acetate, it was important to establish whether brevifoliol itself had this rearranged taxane skeleton, or if the rearrangement could have occurred during the acetylation.

This structure represented a departure from the existing naturally occurring taxane structures available at that time, previously seen only as a product of rearrangement under strongly acidic conditions (Samaranayake *et al.* 1990). Crystallography of the original compound was not done because it failed to yield adequate crystals for analysis without prior acetylation. This made it necessary to determine whether this new ring structure was naturally occurring, or formed during the acetylation.

In one such ring contraction, taxol underwent rearrangement of the A-ring, accompanied by dehydration, to produce an isopropenyl group at C-1, as well as other changes such as the opening of the oxetane ring. Since the ^{13}C NMR spectra of both brevifoliol and its monoacetate showed these signals at δ 62.4 assigned to the C-1

carbon and the one at δ 75.9 assigned to the quaternary C-15 containing tertiary hydroxyl, it appeared unlikely that such a rearrangement took place during the acetylation. HETCOR and APT experiments corroborated these conclusions, thereby agreeing with the structure determined by the x-ray crystallographic method.

Further analysis of the ^{1}H COSY spectrum revealed an isolated spin system of two doublets due to H-9β at δ 6.05 and H-10α at δ 6.53, with a pseudo-axial orientation indicated by the degree of splitting (J=10.6 Hz); and significant broadening of the signal at δ 6.05. Some amount of the deshielding of H-10α relative to H-9β was expected, due to the adjacent double bond, which makes the C-10 position allylic. The presence of a benzoate at this position would be expected to cause a further downfield shift based on analogous compounds already known (Chu *et al.* 1992). With a thorough analysis of the ^{1}H NMR and ^{1}H COSY spectra, the signal at δ 4.38 (t, 7.6 Hz) was assigned to the H-13β proton, which coupled strongly with H-14β at δ 2.46 (dd, 14.0,7.6 Hz.); as well with H-14α at δ 1.25 (dd, 14.0, 7.6 Hz). Weak long range coupling to the C-18 methyl protons at δ 2.01 was also evident, as the slight broadening of this peak is generally attributed to this long range coupling in other taxanes.

The isolated spin system of H-5β, H-6α, H-6β and H-7α is easily identified in most taxanes, with a tendency to show a sharp multiplet for H-7α and broader, poorly resolved splitting for H-5β, especially if H-5β is not esterified (Della Casa de Marcano & Halsall, 1970; Rao *et al.* 1995). The H-5β broad singlet at δ 4.45 interacts with the H-6α multiplet at δ 1.86, which interacts with the H-6β multiplet at δ 2.02, which in turn interacts with the H-7α signal at δ 5.56 (dd, 5,11 Hz.). In many cases esterification of a hydroxyl causes a deshielding effect on the related proton of about 1 ppm. The chemical shifts and splitting patterns indicated that the acetate groups were at C-7 and C-9, with the benzoate at C-10.

The remaining carbons are the 4 methyl groups usually seen in taxanes on C-15 (methyl 16 and methyl 17); at C-12 (methyl 18) and at C-8 (methyl 19). The methyl group located on the 11/12 double bond (methyl 18) is often quite deshielded in the proton spectrum (δ 2.01, s) but shielded in the carbon spectrum (δ 12.0). This usually aids in its assignment along with further evidence from Heteronuclear NMR experiments. Methyl 19 is usually shielded in both the ^{1}H (δ 0.90, s) and ^{13}C (δ 12.9, q) spectra, as seen here.

This class of compounds commonly referred to as 11(1→15)-*abeo*-taxanes or occasionally A-nortaxanes. Many compounds of this type are now known, some containing the 4/20 unsaturation as in brevifoliol and others with a 4/20 oxetane structure as seen in 11(1→15) *abeo* baccatin VI.

Experimental

Extraction of the Needles of *Taxus brevifolia*

The needles obtained from a supplier (Mr. Patrick Connolly, Yew Wood Industries, 6928 North Interstate Avenue, Portland, OR 97217) were air-dried for one week. The dried needles (20 Kg) were extracted by immersing in methanol at room temperature. After two days, the extract was drained, concentrated under reduced pressure at temperatures below 35 ° C. The recovered methanol was reused for a second extraction, which was processed the same way. After two more extractions, the combined concentrate was freed from some more of the methanol to obtain a dark green syrup.

The above syrup was partitioned between water (10 gallons) and chloroform (10 gallons). The organic layer was separated and the extraction carried out twice more

using 5 and 3 gallons respectively. The combined chloroform extract was concentrated under reduced pressure to reach a dark green semi-solid stage (800-900g).

Reverse Phase Column Chromatography:

The column used was a threaded glass column of the Mitchell-Miller type (2.5 x 24") with the appropriate fittings, purchased from Ace Glass Co., Vineland, NJ suitable for low pressure liquid chromatography. A slurry of the C-18 bonded silica (800 g) (Spherisorb, 15-35 micron diameter) purchased from, Phase Separations Inc., Norwalk, CT) in methanol was poured into the column, which was run under a gentle pressure by using a metering pump (Fisher/Eldex) until an adequately packed bed was obtained. The column was then equilibrated with 25% acetonitrile in water, to prepare for the addition of the sample.

The extract solids (200 g) was dissolved in acetonitrile (400 ml) by warming to make sure that no lumps remained. To this was added approximately 200 g equivalent of the equilibrated resin (about 20% of the column packing) with stirring. As the stirring continued, the slurry was diluted with 25% acetonitrile in water (500 ml); followed by water to make up a total volume of approximately 2 L. The stirring was continued with occasional warming to 50-60 $^{\circ}$ C for about 15 min. At this point, a sample of the slurry taken into a test tube, showed that the silica settled readily to give a clear supernatant and no green precipitate or oily material was present. The slurry was then filtered using light suction and the solid (silica with the sample) re-slurried using part of the filtrate and the thick slurry added to the column. The rest of the clear supernatant was then pumped on to the top of the column using the metering pump. From time to time, the column feed was checked to see that it remained clear, and if not, to either warm briefly or add minimal amounts of acetonitrile to it until it became clear, so as to prevent precipitate from appearing and blocking the pump.

After the sample addition was completed, fresh 25% acetonitrile/ water was passed through, followed by the step gradient of acetonitrile/ water (30, 35, 40, 45, 50 and 60%) was used. Fractions (200 ml) were collected and monitored by UV absorbance (at 275 nm), TLC and analytical HPLC. The change to the next concentration of solvent was determined by the results of monitoring the fractions. For example, when the absorbance values rose as a result of the previous change, the solvent was continued until a definite trend to lower values was seen. Similarly, when the TLC showed the trend towards decreasing intensity of the major spot, and no new spot had shown a tendency to increase, the solvent was changed to the next level. In general, 2-3 multiples of the hold-up volumes of the column were used.

After the elution with the 60% solvent was completed, the column was washed with 100% methanol, followed by a mixture of methanol/ ethyl acetate/ ligroin which stripped the column of the chlorophylls, waxes and other lipid soluble components. After this solvent, washing with methanol and equilibration with 25% acetonitrile/ water made the column ready for another run.

After the monitoring, fractions with low UV-absorbance values were combined and concentrated into groups, based on the TLC data. Those fractions with relatively stronger UV readings were let stand at room temperature for 3-5 days, whereby crystals appeared in several sections of the fraction sequence. These crude crystals were filtered, dried and purified further either by recrystallization or using a small silica column (normal phase).

Brevifoliol [3-1]

The fractions containing this component gave crystals but only a small portion was obtained in this form. Hence, after filtration of the crude crystals, the filtrate was concentrated to dryness and the solid taken up in dichloromethane and passed through

a column of normal phase silica, using a ratio of 3-5 g of silica per gram of the solid. The effluent and washes which contained the compound were combined, concentrated to dryness and the solid crystallized from a mixture of acetone and ligroin to obtain brevifoliol as a colorless crystalline solid, yield from 200 g of the chloroform extract solids, 12 g, 0.25% of the dried needles. $[\alpha]_D^{23}$ -27 ° ($CHCl_3$; c 1.03); m.p. 220-222 ° C (lit. 200-203 ° C [Balza *et al.* 1991][44]);

FAB-MS *m/z*: 557 $[MH]^+$, 539 $[MH-H_2O]^+$, 479 $[MH-AcOH]^+$, 435 $[MH-PhCO_2H]^+$, 417 $[MH-PhCO_2H-H_2O]^+$, 375 $[MH-PhCO_2H-AcOH]^+$, IR (KBr) ν_{max} cm^{-1}: 3370, 1740, 1650, 1600, 1585, 1450, 1370, 1265, 1180. UV λ_{max} log ε 3.01 (269 nm); log ε 4.32 (223 nm).

^{1}H NMR (600 MHz, $CHCl_3$, δ) Table 3-1: 0.90, s (H-19); 1.05, s (H-16); 1.30 (dd, J=7.2, 13.8 Hz, H-14α); 1.35, s (H-17); 1.50 (d, J=14.1 Hz, H-2α); 1.76 (s, methyl, 9-acetate); 1.80 (m, H-6α); 2.0 (m, H-6β); 2.01 (s, H-18); 2.07 (s, 7-acetate methyl); 2.36 (dd, J=14.1, 9.6 Hz, H-2β); 2.46 (dd, J=7.2, 13.8 Hz, H-14β); 2.67, br s (C-15 OH, exchangeable with D_2O); 2.77 (br d, J=9 Hz, H-3α); 4.38 (t, J=7.2 Hz, H-13β); 4.43 (br s, H-5β); 4.82, s (H-20 A); 5.18, s (H-20 B); 5.57 (dd, J=4.8, 11.4 Hz, H-7α); 6.05 (poorly resolved br d, J=10.5 Hz, H-9α); 6.53 (d, J=10.5 Hz, H-10β); 7.43 (t, J=7.8 Hz, H-Bz-*meta*); 7.56 (t, J=7.8 Hz, H-Bz-*para*); 7.87 (d, J=7.8 Hz, H-Bz-*ortho*).

^{13}C NMR ($CDCl_3$, 600 MHz, δ) Table 3-2: 12.0 (C-18 methyl, q); 12.9 (C-19 methyl, q); 20.7 (7-O acetate methyl, q) ; 21.4 (9-O acetate methyl, q); 24.8 (C-17 methyl, q); 26.9 (C-16 methyl, q); 29.1 (C-2, t); 36.0 (C-6, t); 37.9 (C-3, d); 45.0 (C-8, s); 47.3 (C-14, dd); 62.4 (C-1, s); 70.3 (C-7, d); 70.9 (C-10, d); 72.4 (C-5, d); 75.9 (C-15, s); 76.7 (C-13, d); 77.1 (C-9, d); 112.0 (C-20, t); 128.7 (C-Bz-*meta,* d); 129.3 (C-Bz-*ipso*, s); 129.4(C-Bz-*ortho*, d); 133.3 (C-Bz-*para*, d); 133.9 (C-12, s); 149.0 (C-4, s); 151.5 (C-11, s); 164.3 (CO-Ph, s); 169.9 (CO-Acetate, s); 170.5 (CO-Acetate, s).

Analysis calculated for $C_{31}H_{40}O_9$: C, 66.89; H, 7.24. Found: C, 67.12; H, 7.35;

Brevifoliol-5-Monoacetate [3-2]

A mixture of brevifoliol (0.2 g); acetic anhydride (2 ml) and pyridine (0.5 ml) was stirred at room temperature for 2-3 min. Water was added to decompose the reagent, and the solid filtered after 15 min. The solid was crystallized from a mixture of acetone and ligroin to obtain the mono acetate as a colorless crystalline solid, yield, 0.18 g; m.p.224-226 °C;

^{1}H NMR ($CDCl_3$, 600 MHz, δ) Table 3-1: 0.91, s (H-19); 1.02, s (H-16); 1.22 (dd, J=7.2, 13.8 Hz, H-14α); 1.33, s (H-17); 1.46 (d, J=14.1 Hz, H-2α); 1.76 (s, 9-O acetate methyl); 1.88 m, 2.0 m (H-6); 2.06 x 2, s (methyl-18, 5-O acetate methyl); 2.08 (s, 7-O acetate methyl); 2.40 (dt, J=14.1, 9.6 Hz, H-2β); 2.42 (dd, J=7.2, 13.8 Hz, H-14β); 2.75 (d, J=9 Hz, H-3α); 2.83, br s (C-15 OH, exchangeable with D_2O); 4.53 (t, J=7.2 Hz, H-13β); 4.90, s (H-20 A); 5.28, s (H-20 B); 5.37 (br s, J= H-5β); 5.65 (dd, J=4.8, 11.4 Hz, H-7α); 6.02 (poorly resolved br d, J=10.5 Hz, H-9α); 6.63 (d, J=10.5 Hz, H-10β); 7.43 (t, J=7.8 Hz, H-Ph-*meta*); 7.56 (t, J=7.8 Hz, H-Ph-*para*); 7.87 (d, J=7.8 Hz, H-Ph-*ortho*).

^{13}C NMR ($CDCl_3$, 600 MHz, δ) Table 3-2: 11.8 (C-18 methyl, q); 12.9 (C-19 methyl, q); 20.8 (7-O acetate methyl, q); 21.2 (5-O acetate methyl, q); 21.4 (9-O acetate methyl, q); 24.8 (C-17 methyl, q); 27.0 (C-16 methyl, q); 29.2 (C-2, t); 33.9 (C-6, t); 38.8 (C-3, d); 44.8 (C-8, s); 47.1 (C-14, dd); 63.0 (C-1, s); 69.7 (C-7, d); 70.7 (C-10, d); 74.1 (C-5, d); 75.6 (C-15, s); 76.9 (C-13, d); 77.9 (C-9, d); 114.0 (C-20, t); 128.7 (C-Ph-*meta,* d); 129.2 (C-Ph-*ipso*, s); 129.4(C-Ph-*ortho*, d); 133.3 (C-Ph-*para*, d); 134.0 (C-11, s); 145.2 (C-4, s); 151.1 (C-12, s); 164.1 (CO-Ph, s); 169.9 X 2(CO-Acetate, s); 170.2 (CO-Acetate, s).

Analysis calculated for $C_{33}H_{42}O_{10}$: C, 66.20; H, 7.07. Found: C, 66.38; H, 7.19.

Brevifoliol-5,13-Diacetate [3-4]

The above reaction was repeated, except that it was heated at 80-90° C (water bath) for 30 min. After cooling, water was added and the solid filtered after 10 min. The solid was crystallized from acetone/ ligroin to give the diacetate as a colorless crystalline solid, yield, 0.2 g; m.p.241-243 °C;

^{1}H NMR ($CDCl_3$, 600 MHz, δ) Table 3-1: 0.92, s (H-19); 1.11, s (H-16); 1.25 (dd, J=7.2, 13.8 Hz, H-14α); 1.35, s (H-17); 1.46 (d, J=14.1 Hz, H-2α); 1.75 (s, 9-O acetate methyl); 1.90 (m, H-6α) 2.0 (m, H-6β); 2.02 (s, 5-O acetate methyl); 2.03 (s, 13-O acetate methyl); 2.07 (s, 18 methyl); 2.08 (s, 7-O acetate methyl); 2.41 (dd, J=14.1, 9.6 Hz, H-2β); 2.51 (dd, J=7.2, 13.8 Hz, H-14β); 2.72 (d, J=9 Hz, H-3α); 2.74, br s (C-15 OH, exchangeable with D_2O); 4.92, s (H-20 A); 5.28, s (H-20 B); 5.39 (br s, J= H-5β); 5.54 (t, J=7.2 Hz, H-13β); 5.61 (dd, J=4.8, 11.4 Hz, H-7α); 6.09 (poorly resolved br d, J=10.5 Hz, H-9α); 6.65 (d, J=10.5 Hz, H-10β); 7.43 (t, J=7.8 Hz, H-Ph-*meta*); 7.56 (t, J=7.8 Hz, H-Ph-*para*); 7.87 (d, J=7.8 Hz, H-Ph-*ortho*).

^{13}C NMR ($CDCl_3$, 600 MHz, δ) Table 3-2: 11.9 (C-18 methyl, q); 12.9 (C-19 methyl, q); 20.7 (7-O acetate methyl, q); 21.0 (13-O acetate methyl, q); 21.2 (5-O acetate methyl, q); 21.4 (9-O acetate methyl, q); 24.8 (C-17 methyl, q); 27.0 (C-16 methyl, q); 29.1 (C-2, t); 33.9 (C-6, t); 38.8 (C-3, d); 44.8 (C-8, s); 44.1 (C-14, dd); 63.0 (C-1, s); 69.6 (C-7, d); 69.8 (C-10, d); 74.1 (C-5, d); 75.6 (C-15, s); 76.9 (C-13, d); 79.3 (C-9, d); 114.3 (C-20, t); 128.8 (C-Ph-*meta,* d); 129.1 (C-Ph-*ipso*, s); 129.5(C-Ph-*ortho*, d); 133.4 (C-Ph-*para*, d); 136.4 (C-11, s); 145.2 (C-4, s); 147.3 (C-12, s); 164.1 (CO-Ph, s); 169.6 (CO-Acetate, s); 169.9 (CO-Acetate, s); 169.91 (CO-Acetate, s); 170.5 (CO-Acetate, s).

Analysis calculated for $C_{35}H_{44}O_{11}$: C, 65.61; H, 6.92. Found: C, 65.68; H, 6.99.

Brevifoliol-13-Ketone [3-6]

A solution of brevifoliol (0.2 g) in benzene was treated with MnO_2 (manganese dioxide, 1 g, Fisher Scientific) and the mixture heated under reflux for 2 hours, at which time, the starting material was consumed and a slightly faster moving product was formed. The mixture was filtered, concentrated and applied to a small silica column (15 g) in dichloromethane. Elution with 1% acetone in dichloromethane gave the major product which was recovered by concentration as a colorless powder, yield, 0.12g. The ^1H- and ^{13}C NMR spectra of this faster moving product were quite poorly resolved and only gave usable results at temperatures below -10 ° C. Recrystallization and further chromatography failed to improve this situation, and low temperature NMR experiments indicated that a rotameric equilibrium was responsible for the poor resolution seen in these spectra.

^1H NMR ($CDCl_3$, 600 MHz, -40 ° C, δ) major rotamer: 0.92, s (H-19); 0.98, s (H-16); 1.35, s (H-17); 1.46 (d, J=14.1 Hz, H-2α); 1.78 (s, 9-O acetate methyl); 1.90 (m, H-6α) 2.0 (m, H-6β); 2.01 (s, 18 methyl); 2.02 (s, 7-O acetate methyl); 2.32 (d, J=19.0, 14α); 2.48 (d, J=19.0 Hz, H-14β); 2.92 (unresolved, H-3α); 2.74, br s (C-15 OH, exchangeable with D_2O); 4.92, s (H-20 A); 5.28, s (H-20 B); 5.39 (br s, J= H-5β); 5.54 (t, J=7.2 Hz, H-13β); 5.61 (dd, J=4.8, 11.4 Hz, H-7α); 6.09 (poorly resolved br d, J=10.5 Hz, H-9α); 6.65 (d, J=10.5 Hz, H-10β); 7.43 (t, J=7.8 Hz, H-Ph-meta); 7.56 (t, J=7.8 Hz, H-Ph-para); 7.87 (d, J=7.8 Hz, H-Ph-ortho).

^{13}C NMR ($CDCl_3$, 600 MHz, δ): 8.9 (C-18 methyl, q); 12.5 (C-19 methyl, q); 20.7 (7-O acetate methyl, q); 21.0 (9-O acetate methyl, q); 26.2 (C-17 methyl, q); 26.6 (C-16 methyl, q); 27.3 (C-2, t); 27.7 (C-6, t); 34.2 (C-3, d); 43.2 (C-8, s); 48.3 (C-14, t); 58.1 (C-1, s); 70.8 (C-7, d); 71.0 (C-10, d); 73.2 (C-9, d); 75.6 (C-15, s); 111.4 (C-20, t); 128.8 (Ph-meta, d); 129.1 (Ph-ipso, s); 129.5(Ph-ortho, d); 133.4 (Ph-para, d); 136.4 (C-11, s);

145.2 (C-4, s); 144.3 (C-12, s); 163.1 (C-11, s); 165.4 (CO-Ph, s); 169.3 (CO-Acetate, s); 170.3 (CO-Acetate, s); 207.5 (C-13 Ketone, s).

Analysis calculated for $C_{31}H_{38}O_9$: C, 67.13; H, 6.91. Found: C, 67.48; H, 6.97.

Dihydrobrevifoliol [3-7]

A solution of brevifoliol (0.2 g) in ethyl acetate (10 ml) was hydrogenated in a Parr apparatus using Platinum oxide (0.05 g) for 16 hours. TLC revealed the formation of a slightly slower moving product. The mixture was filtered and the filtrate concentrated to dryness and purified by chromatography on silica gel in dichloromethane. Elution with 2% acetone in dichloromethane gave a minor product, which was not further investigated. The fractions eluted with 2-5% methanol in dichloromethane gave the major product, which was obtained as a colorless powder, yield, 0.1 g,

Reduction of the 4(20) double bond resulted in significant broadening of most peaks in the ^{1}H- and ^{13}C NMR spectra, but the appearance of additional signals from methyl group at C-20 and methylene at C-4 could be seen, as well as the loss of the two characteristic exocyclic methylene singlets.

Analysis calculated for $C_{31}H_{42}O_9$: C, 66.60; H, 7.60. Found: C, 66.89; H, 7.88.

Brevifoliol Epoxide [3-8]

A mixture of brevifoliol (0.3 g) and meta-chloroperoxybenzoic acid (MCPBA, 0.2 g) in toluene (15 ml) was heated under reflux for 30 min. After cooling, the mixture was diluted with ether, washed successively with aqueous sodium bisulfite, aqueous sodium bicarbonate and saline, and the organic layer concentrated to dryness. The solid was crystallized from acetone/ligroin, to give a colorless crystalline epoxide, yield, 0.15 g; m.p. 227-230 °C.

^{1}H NMR ($CDCl_3$, 600 MHz, -40^0 C, δ) : 1.01 (H-19, s); 1.09 (H-16, s); 1.24 (dd, J=7.2, 13.8 Hz, H-14α); 1.27 (H-17, s); 1.40 (H-2α, br d J=14.1 Hz); 1.76 (s, methyl, 9-acetate); 1.80 (m, H-6α); 2.0 (m, H-6β); 2.01 (s, H-18); 2.07 (s, 7-acetate methyl); 2.36 (dd, J=14.1, 9.6 Hz, H-2β); 2.46 (dd, J=7.2, 13.8 Hz, H-14β); 2.67, br s (C-15 OH, exchangeable with D_2O); 2.64 (H-3α, br d, J=9 Hz); 2.72 (C-20, s); 3.59 (C-20, s); 4.20 (br s, H-5β); 4.46 (H-13β, br d, J=7.2 Hz); 5.57 (H-7α, br d, J=4.8, 11.4 Hz); 6.05 (H-9α, poorly resolved br d, J=10.5 Hz); 6.54 (H-10β, br d, J=10.5 Hz); 7.43 (Ph-meta, t, J=7.8 Hz); 7.56 (Ph-para, t, J=7.8 Hz); 7.87 (Ph-ortho, d, J=7.8 Hz).

^{13}C NMR ($CDCl_3$, 600 MHz, -40^0 C, δ) : 11.9 (C-18 methyl, q); 12.9 (C-19 methyl, q); 20.7 (7-O acetate methyl, q) ; 21.3 (9-O acetate methyl, q); 23.9 (C-2, t); 25.0 (C-17 methyl, q); 27.1 (C-16 methyl, q); 34.1 (C-3, d); 34.4 (C-6, t); 45.4 (C-8, s); 46.4 (C-14, t); 50.1 (C-4, s); 60.2 (C-20, t); 62.4 (C-1, s); 69.5 (C-7, d); 70.5 (C-10, d); 71.7 (C-5, d); 75.8 (C-15, s); 76.7 (C-13, d); 77.1 (C-9, d); 128.7 (Ph-meta, d); 129.4 (Ph-ipso, s); 129.5(Ph-ortho, d); 133.2 (Ph-para, d); 134.3 (C-12, s); 151.3 (C-11, s); 164.3 (CO-Ph, s); 169.96 (CO-Acetate, s); 170.0 (CO-Acetate, s).

Analysis calculated for $C_{31}H_{40}O_{10}$: C, 65.02; H, 7.04. Found: C, 64.72; H, 7.24.

Ozonization of Brevifoliol: Brevifoliol-norketone [3-9]

A solution of brevifoliol (1 g) in a 9:1 mixture of chloroform and methanol (25 ml) was cooled in a dry ice/ acetone bath and saturated with ozone produced by an ozonizer (Ozone Research and Equipment Co., Phoenix, AZ). After testing for the absence of the starting material by TLC, the mixture was removed from the bath and treated with dimethyl sulfide (1 ml) and let stand at room temperature for 2 h. It was then concentrated to dryness and applied to a silica column prepared in chloroform. Elution with 2% acetone in chloroform gave two bands, which were separated and the fractions concentrated separately.

The faster moving fraction [3-9] was obtained as a colorless, amorphous powder, yield, 0.25 g.

^{1}H NMR ($CDCl_3$, 600 MHz, -40 ° C, δ) major rotamer: 1.02, s (H-19); 1.05, s (H-16); 1.35, s (H-17); 1.46 (d, J=14.1 Hz, H-2α); 1.78 (s, 9-O acetate methyl); 1.98 (m, H-6α); 2.0 (m, H-6β); 2.01 (s, 18 methyl); 2.02 (s, 7-O acetate methyl); 2.32 (d, J=19.0, 14α); 2.48 (H-14β, dd, J=14, 7.5 Hz); 3.19 (H-3α, d, J=10.2); 4.20 (H-5β, br s); 4.48 (H-13β, br t, J=7.2 Hz); 5.78 (H-7α, br m); 5.92 (H-9α, br); 6.52 (H-10β, br d, J=10.5 Hz); 7.43 (Ph-meta, t, J=7.8 Hz); 7.56 (Ph-para, t, J=7.8 Hz);7.87 (Ph-ortho, d, J=7.8 Hz).

^{13}C NMR ($CDCl_3$, 600 MHz, δ): 12.1 (C-18 methyl); 14.2 (C-19 methyl); 20.6 (7-O acetate methyl); 21.2 (5-O acetate methyl); 21.3 (9-O acetate methyl); 24.8 (C-17 methyl); 27.0 (C-16 methyl); 29.1 (C-2); 33.9 (C-6); 34.8 (C-3); 46.4 (C-8); 46.4 (C-14); 62.0 (C-1); 69.9 (C-7); 70.7 (C-10); 72.0 (C-5); 75.9 (C-15); 76.9 (C-13); 77.3 (C-9); 128.8 (Ph-meta); 129.1 (Ph-ipso); 129.5(Ph-ortho); 133.4 (Ph-para); 134.4 (C-11); 144.5 (C-4); 147.3 (C-12); 164.3 (CO-Ph, s); 169.8 (CO-Acetate, s); 170.0 (CO-Acetate, s);208.0 (C-13 C=O).

Analysis calculated for $C_{30}H_{38}O_{10}$: C, 64.50; H, 6.86. Found: C, 64.68; H, 6.97.

The slower moving fraction was obtained as a colorless crystalline solid, yield, 0.3 g; m.p.225-232 ° C. It was found to be identical with the epoxide [3-8], described above.

Brevifoliol-4,20-Diol [3-10]

To a solution of brevifoliol (0.4 g) in pyridine (10 ml) was added osmium tetroxide (0.2 g) and the reaction mixture stirred for 1 h, after which time, the starting material was replaced by a much slower moving component. After decomposing the excess reagent with a solution of sodium bisulfite in pyridine, water and dilute sulfuric acid were added and the mixture extracted with dichloromethane. After concentration, the product was

placed on a silica column in dichloromethane. Elution with 2% methanol in dichloromethane gave the major band which yielded [3-8] as a white powder, final yield, 0.12 g.

Analysis calculated for $C_{31}H_{42}O_{11}$: C, 63.04; H, 7.17. Found: C, 62.88; H, 7.25.

Saponification of Brevifoliol [3-11]

A solution of brevifoliol (1 g) in methanol (20 ml) was stirred with 1N potassium hydroxide (10 ml) for 1 h. TLC showed that the starting material was absent and very slow moving, non UV-absorbing component produced. The reaction mixture was passed through a small column or Amberlite-IR120 (a sulfonic acid resin) in the H+ form. The column was washed with 1:1 methanol/water. The effluent and washes were concentrated to dryness and the solid crystallized from acetone to give [3-11] as a colorless crystalline solid, yield, 0.45 g; m.p. 290 °C dec.

Analysis calculated for $C_{20}H_{32}O_6+H_2O$: C, 62.15; H, 8.87. Found: C, 62.48; H, 8.99.

Debenzoyl Brevifoliol-Pentaacetate [3-12]

Compound [3-11] (0.2 g) was acetylated using acetic anhydride (2 ml) and pyridine (0.5 ml) by heating at 80 ° C for 30 min. Water was added to decompose the reagent and the solid filtered. It was crystallized from ether/ ligroin, yield, 0.2 g; m.p. 184-187 ° C.

^{1}H NMR ($CDCl_3$, δ): 6.36 (H-10, d, J=10.2); 5.88 (H-9, br d, 10.2); 5.54 (H-13β, t, J=7.2); 5.53 (H-7α, q, J=5.4, 10.2); 5.36 (H-5β, br s); 5.26 (H-20b, s); 4.87 (H-20a, s); 2.65 (OH-15, s); 2.64 (H-3α, d, J=9); 2.48 (H-14β, dd, J=13.8, 7.2); 2.36 (H-2α, dd, J=14.1, 9.3); 2.07 (AcMe, s); 2.06 (AcMe, s); 2.02 (AcMe, s); 2.00 (AcMe, s); 1.97 (AcMe, s); 1.95 (18-Me, s); 2.0 (H-6α, m); 1.85 (H-6β, cm); 1.42 (H-2β, d, J=14.4); 1.31 (H-17 Me);1.22 (H-14α, dd, J=13.8, 7.2); 1.13 (H-16 Me); 0.88 (H-19 Me).

IR *v* max (KBr, cm^{-1}): 3565 (OH, sharp); 2960, 1740 (C=O); 1370, 1230, 1030.

MS(FAB): 580 [MH+].

Periodate Oxidation of [3-11] to [3-13]

A solution of [3-11] (0.2 g) in methanol (5 ml) was treated with sodium periodate (0.3 g) in 1N sulfuric acid (2 ml). After 30 min, TLC showed that the starting material was absent and was replaced by a faster moving product visible under the UV light, unlike the starting material. After dilution with water, the mixture was extracted with ethyl acetate and the extract concentrated to dryness. The crude dialdehyde [5-13] was not further purified before the next reaction, but did exhibit signals for two aldehydes the ^1H spectra. The only significant changes from the parent compound showed the loss of the isolated ^1H spin system from the protons on C-9 and C-10, and the conversion of two hydroxyl carbons into aldehydes (Guthrie, 1961).

^1H NMR ($CDCl_3$, δ) : 9.96 (CHO, s); 9.42 (CHO, s); 5.36 (H-20b, s); 5.02 (H-20a, s); 4.58 (H-5α, m); 4.40 (H-13α, br t); 2.60 (H-3β, d); 1.30 (Me, s); 1.25 (Me, s); 1.02 (Me, s); 0.88 (Me, s).

Analysis calculated for $C_{30}H_{42}O_{11}$: C, 62.27; H, 7.32. Found: C, 62.10; H, 7.44.

Formation of Osazone [3-14] from [3-13] with 2,4-DNPH

Dialdehyde [3-13] was dissolved in methanol, (2 ml) and heated with a solution of 2,4-dinitrophenyl hydrazine (0.1 g) in 2N hydrochloric acid (2ml) and methanol (2 ml). After 2 h at room temperature, the mixture was extracted with chloroform and the concentrated extract chromatographed on a silica column. The major band [3-14] was obtained as an orange yellow crystalline solid, m.p. 215-218 °C. Both ^1H- and ^{13}C NMR spectra indicated the retention of all twenty of the carbons from the *abeo*-taxane skeleton.

Debenzoyl Brevifoliol [3-15]

A solution of brevifoliol (0.3 g) in 30% methanol in water (10 ml) was heated in a sealed tube at 135 ° C for 90 min. The cooled mixture was neutralized with sodium bicarbonate and extracted with chloroform. The extract was purified by chromatography on a C-8 reverse phase column using a step gradient of 25-60% acetonitrile in water in 5% increments of solvent concentration. Elution with 30-35% acetonitrile in water gave the major product, which was obtained as a white powder, yield, 0.1 g. The ^{1}H and ^{13}C NMR spectra revealed the loss of the benzoate from the C-10 position and retention of the acetate substituents at C-9 and C-7

Analysis calculated for $C_{24}H_{36}O_8$: C, 63.70; H, 8.02. Found: C, 63.89; H, 7.88.

Benzoic acid was also isolated from the reaction mixture and confirmed using NMR and UV analyses.

CHAPTER 4
SOME UNUSUAL REACTIONS OF BREVIFOLIOL

The isolation and structural elucidation of brevifoliol [3-1] was described in detail in Chapter 3. Brevifoliol occurs to the extent of 0.2-0.3 % in the needles of *T. brevifolia*, and to a lesser extent in the bark of the same species, in the needles of *T.* x *media* Hicksii (Rehd.) and in the needles of *T. wallichiana*. (Georg *et al.* 1993). Large quantities of the crystalline compound can be readily isolated from the needles of *T. brevifolia*, which is the best source for the compound.

However, other than acetylation to a diacetate (Balza *et al.* 1991); and the attachment of the N-benzoyl phenyl-isoserine side chain at the C-13 position (Georg, *et al.* 1993); no record of its reactions reflecting its various functional groups has been published. This paucity of such information is in keeping with the current trend that, in spite of the virtual explosion of new taxanes that were isolated and characterized structurally by spectral data over the past five years. Very few have been investigated for their chemical reactions, for the relative reactivities of similar functional groups, or for any unusual reactions as a consequence of their stereochemical disposition.

Understanding the products of the various transformations that a compound can be subjected to and the relative rates of reaction can lead to important insight concerning entity, and for the general advancement of chemistry. Although the structure of brevifoliol is now well established as a result of spectral and x-ray crystallographic data, we can gain insight into this molecule through reactions such as those described in Chapter 3. In addition to these, a number of reactions also carried out for the purpose of

structural elucidation of [3-1] took unexpected courses. The products of these reactions were isolated and characterized structurally, and the details are described here.

1. Acid-Catalyzed Acetylation

In Chapter 3, acetylation of [3-1] by means of acetic anhydride and pyridine to give the crystalline monoacetate [3-2] and the diacetate [3-4] has been described. We isolated from the needles of *T. brevifolia* another taxane, which resembled brevifoliol, giving a dark greenish blue color when sprayed with sulfuric acid and heated. This same product was also subsequently isolated by others (Barboni *et al.* 1993). It was similar to but different from the monoacetate [3-2], but when acetylated, gave [3-4], thus showing that it was the 13-acetate [3-3].

Acetylation of brevifoliol using acetic anhydride and BF_3 gave a different crystalline product, with almost the same R_F as that of the diacetate [3-4]. The BF_3 product closely resembled the 5, 13-diacetate in its NMR spectral properties also, but with some differences. In the ^1H NMR spectrum, the singlet at δ2.74 which is assigned to the 15-hydroxyl in the diacetate [3-4], was no longer present. The two methyl singlets assigned to C-16 and C-17 at δ1.11 and δ1.35 were deshielded to δ1.63 and δ1.71. The other two (C-18 and C-19) showed either no shift or a much smaller one (δ 2.03$\rightarrow\delta$ 1.96 for C-18). Thus, the significant downfield shift of the signals due to C-16 and C-17 suggested that acetylation of the 15-hydroxyl might have taken place.

Additionally, the signals due to the H-2α and 2β showed slight downfield shifts (H-2α: δ1.46, broad doublet, J=13 Hz to 1.53 ppm; H-2β: δ2.41, doublet of doublets, J=9,13 Hz to 2.65 ppm). The spectra of brevifoliol and its acetates generally show the H-10α signal as a sharp and well resolved doublet, but the one due to H-9β as a broad, poorly resolved doublet. In the BF_3-reaction product, the signal due to H-9β was not only a well resolved doublet (J=10.8 Hz); but also shielded by 0.29 ppm to δ 5.80. These

observations suggest that the C-15 hydroxyl might be responsible for the blurring of the signal of H-9β, and acetylation of this hydroxyl has eliminated that interaction.

Further support for the acetylation having taken place at the C-15 hydroxyl is shown by the appearance of another acetyl methyl signal at δ 2.11 in the ^{1}H NMR spectrum. The appearance of two more signals in the ^{13}C NMR spectrum was also evident, in which 5 acetyl methyl and 5 acetyl-carbonyl signals were present, with the fifth one at δ 21.7 and δ 169.5, respectively. Also in the ^{13}C NMR spectrum, a significant downfield shift from δ 75.6 to δ 87.2 for the C-15, and an upfield shift from δ 27.0 to δ 24.8 and from δ 23.1 to δ 22.0 for the signals due to C-16 and C-17 respectively, completes the evidence to indicate that the 15-hydroxyl was acetylated to give [3-5].

A comparison of the ^{13}C NMR spectral data of brevifoliol, the 5-monoacetate [3-2], the 13-monoacetate [3-3] (naturally occurring and confirmed through semi-synthesis); the 5, 13-diacetate [3-4] and of the BF_3-catalyzed acetylation product [3-5] were shown in Chapter 3 in Table 3-1.

2. Oxidation

Oxidation of brevifoliol with manganese dioxide gave the monoketone, the NMR spectral data of which showed that the 13-hydroxyl was oxidized, leading to the structure [3-6], as described in Chapter 3. Oxidation of [3-1] with Jone's reagent gave initially, the same 13-monoketone [3-6], but on further reaction, this was replaced by a faster moving compound [4-1], whose spectral data pointed to an unexpected course of reaction.

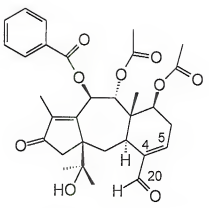
The molecular formula of the product, $C_{31}H_{36}O_9$ (MH+, 553) indicated the loss of 4 protons, as compared to brevifoliol. Although this might indicate that both hydroxyls were oxidized to give the diketone [4-2], certain features suggested otherwise. To begin with, the ^{1}H NMR spectrum showed broad peaks which indicated the existence of a

rotameric equilibrium, which was confirmed by a spectrum taken at –40 ° C, in which two sets of peaks with a 5:1 ratio were seen.

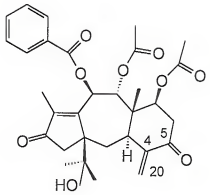
In the major rotamer, the coupling between the C-9 and C-10 protons was found to be 4 Hz, in contrast to the value of 10.5 Hz shown by brevifoliol [3-1], (a similar diketone prepared from 2-acetoxy brevifoliol (taxchinin A); described by Fuji *et al.* (1992) and Appendino *et al.* (1993) also showed a coupling of 4 Hz. Next, a singlet appeared at 9.4 ppm, which interacted in the HETCOR spectrum with the peak at 194 ppm. The latter showed a negative signal in the Attached Proton Test (APT). These observations indicated the presence of an aldehyde functionality, presumably at C-20. Additionally, in the spectrum of the diketone such as [4-2], the C-5 proton signal was absent, as expected, and the exocyclic methylene protons appeared as two singlets at δ 5.06 and δ 5.94 ppm. The corresponding carbon signals appearing at δ127 and δ143.4 ppm.

However, in the Jones oxidation product, the singlets due to the exocyclic methylene protons were absent, and the characteristic C-20 carbon signal which appears in the δ110-δ120 ppm region was also missing. Instead, a signal was found at δ 6.73 ppm, which interacted with the signals at δ2.5 and δ2.7 (C-6 protons); and in the HETCOR spectrum, with the signal present at δ 147 ppm, and this latter gave a negative signal in the APT spectrum. These data seem to suggest that the product is not the 5,13-diketone [4-2], but an aldehydic product with a double bond present at C-4/C-5, as shown in [4-1].

One possible explanation is that the sulfuric acid in the reagent caused hydration of the 4/20 double bond, and the primary alcohol so generated was oxidized to the aldehyde, followed by dehydration to yield the 4/5-double bond.



[4-1] - Jones Oxidation - Aldehyde



[4-2] - MnO_2 Diketone

Figure 4-1 : Oxidation Products

3. Action of BF_3 on Brevifoliol [4-3]

Before the structure of brevifoliol was fully established, the possibility that the two hydroxyls present in the compound might be vicinal to each other was considered. With the aim of forming an isopropylidene derivative, brevifoliol was reacted with acetone in the presence of Dowex-50 (sulfonic acid resin) as the catalyst. Reaction took place readily with the formation of a faster moving product, with some decomposition also taking place (colors). The reaction proceeded with less decomposition when BF_3-etherate was used as the catalyst.

Chromatography of the mixture from either reaction yielded the major product as a colorless crystalline solid. Its NMR spectrum quickly ruled out the possibility of its being an isopropylidene derivative. The FAB-mass spectrum gave a value for the MH+ *m/z* of 481, as compared with 557 for brevifoliol, thus showing a loss of 76 mass units. The elemental analysis, which agreed with $C_{26}H_{32}O_7$ showed a loss of $C_3H_8O_2$ compared to brevifoliol. This may be interpreted as the loss of the C_3H_7O- side chain attached to C-1, as well as that of H_2O, possibly through the elimination of the C-13-OH (or C-5-OH). The evidence to support this assertion is given below.

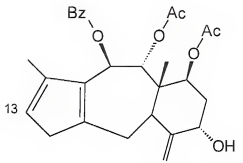
1. The most striking evidence is that only two of the four methyl signals that are seen in brevifoliol appear in the BF_3-reaction product. This is not only observed in the ^1H spectrum, but also in the ^{13}C NMR thus showing that the oxy-isopropyl chain at C-1 is not present.
2. An examination of the isolated spin system: 5β - 6α- 6β - 7α in the ^1H NMR spectrum of brevifoliol and that of the product of BF_3 reaction indicated that the free hydroxyl at C-5α was still present, but that the C-13α-OH was absent. Through a study of the interactions in the COSY spectrum, it was possible to assign (and distinguish) the signals for C-6 and C-14 in the region below δ3.
3. A new methine proton signal appeared as a broad singlet at δ5.84, assigned to H-13, which interacted in the HETCOR spectrum with the signal at δ124.1, which corresponds to C-13.
4. The DEPT spectrum showed the presence of four signals for methyl-type (CH_3) carbons (δ11.4, δ 13.5 for C-19 and C-18, δ 20.7, δ 21.2 for the two CO-Me); two signals for methylene-type (CH_2) carbons (δ 112.4 for C-20, δ 44.6 for C-14, δ 34.1 for C-6 and δ 27.9 for C-2, nine signals for methine-type (R_3CH) carbons (δ133.0, δ129.7, δ128.4 for the five aromatic carbons, four aliphatic CH-OR type carbons [δ73.2 (C-9); δ72.5 (C-5); δ79.6 (C-7); δ67.4 (C-10)], one aliphatic methine type carbon (δ39.6 (C-3)); one unsaturated methine type carbon (δ124.1 (C-13) and nine quaternary carbons (three carbonyls, one aromatic C carrying the carboxyl, C-1(?); C-4, C-11, C-12 and C-8); together, account for the 28 carbons present..
5. HETCOR interactions supported the assignments for the o, m, p- positions in the benzoate, and for the C-13, C-20, C-9, C-5, C-7, C-10, C-3, C-6, C-2, C-18, C-19 and the two acetate methyls.

6. In the 1D nOe-difference spectrum, the interaction between the C-14α,β and C-13 β protons was the strongest. Crowding of the region around δ2.8 ppm made the spectrum more difficult to interpret, and not very informative.

Table 4-1 : NMR Spectra of Compound [4-3] from BF_3 Reaction

Position	Proton	Carbon	APT	DEPT
1	----	145.9	↑	C
2 α	1.96 m	28.0	↑	CH_2
2 β	3.00 m	----	----	----
3 α	2.70 m	39.4	↓	CH
4	----	150.4	↑	C
5 α	4.42 br s	72.6	↓	CH
6 α	1.76 m	34.1	↑	CH_2
6 β	2.05 m	----	----	----
7 α	5.48 br d	70.6	↓	CH
8	----	46.6	↑	C
9 β	5.34 d 6.0	73.2	↓	CH
10 α	6.30 d 6.0	67.5	↓	CH
11	----	146.4	↑	C
12	----	134.2	↑	C
13 β	5.83 br s	124.0	↓	CH
14 α, β	2.85-3.0 cm	44.6	↑	CH_2
18	2.06 s	11.4	↓	CH_3
19	1.23 s	13.4	↓	CH_3
20 A	4.98 br s	112.2	↑	CH_2
20 B	5.20 br s	----	----	----
$\underline{C}O\text{-}C_6H_5$	----	165.2	↑	C
Bz-ipso	----	130.2	↑	C
Bz-ortho	8.01, d 7.5	128.4	↓	CH
Bz-meta	7.44, t 7.5	129.6	↓	CH
Bz-para	7.56, t 7.5	132.9	↓	CH
$CO\underline{C}H_3$	1.98 s 2.00 s	20.6 21.1	↓ ↓	CH_3 CH_3
$\underline{C}OCH_3$	----	169.9 170.4	↑ ↑	C C

^1H NMR were recorded at 600 MHz and ^{13}C NMR at 150 MHz in $CDCl_3$ on a Varian Unity 600 spectrometer at ambient temperature. Chemical shifts δ (ppm) are reported relative to TMS as an internal standard.



[4-3] - BF_3 -etherate Product

Figure 4-2 : BF_3–etherate Catalyzed Elimination Product

Based on this reasoning, the structure of the BF_3-reaction product was assigned as shown [4-3] in Figure 4-2 above. The DEPT spectra of [4-3] are given in Figure 4-3.

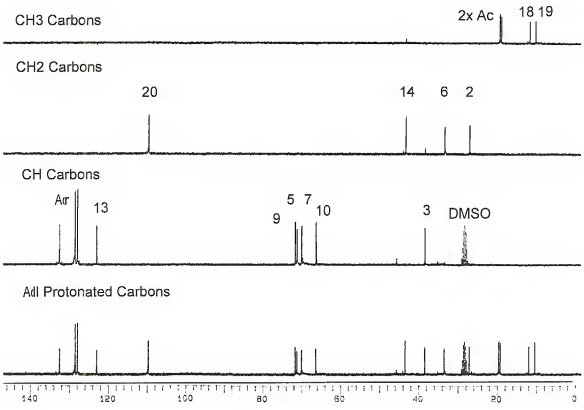


Figure 4-3 : DEPT Spectra of BF_3 Elimination Product [4-3]

A reaction such as this has not been reported in the taxane series, resulting in the loss of the oxy-isopropyl side chain. In taxol and related compounds containing the conventional taxane skeleton, action of Lewis acids such as BF_3 was studied and is shown to produce one or two different changes, depending on the whether protic or aprotic solvent is used. In one case, isomerization of the A-ring from a 6- to a 5-membered A ring takes place with the oxy-isopropyl group attached at C-1. In the second instance, the oxetane ring is opened to form a diol, or with the acetate group migrating from C-4 to C-20 to give the 4-hydroxy-20-acetoxy compound. The reaction described here appears to be a continuation of the action of the Lewis acid on the 5-membered A ring, with the elimination of the oxy-isopropyl substituent.

4. Reaction with Iodine/Silver Acetate [4-4]

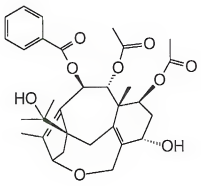
Once the structure of brevifoliol had been elucidated, the value and usefulness of this relatively abundant compound in the needles of T. brevifolia was considered. Since the addition of the N-benzoyl isoserine side chain at C-13 did not generate activity in the final product, it was reasoned that the oxetane ring at the 4/20 position might be necessary to produce activity. To this end, one approach was investigated, involving the use of iodine in some form to add across the 4/20 double bond and thereby permitting substitution with other groups.

Brevifoliol was found to react readily with bromine, but the reaction yielded multiple products and considerable decomposition. Reaction with iodine was similarly complex and led to much decomposition and dark colored products. With the idea that addition of a silver salt which can remove the acid that might be produced, but not be too strongly basic (e.g. silver oxide) and hence hydrolyze the ester functions, silver acetate was selected for use with the iodine. The remote possibility that silver acetate might

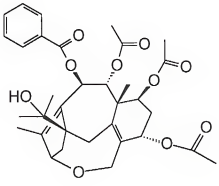
displace the iodine located at C-4 to produce the 4-acetoxy-20-iodo compound was also attractive (Woodard & Brutcher, 1958).

When brevifoliol was stirred with iodine and silver acetate, the course of the reaction was clearly different. No multiple products or decomposition to dark colored products was seen, even when the reaction was continued for 15-30 hours, unlike the reaction with iodine alone that became dark in 1-2 hours. The reaction was continued until the starting material was consumed and a major, faster moving compound was produced. Chromatography on a silica column gave a colorless crystalline solid.

Acetylation with acetic anhydride and pyridine at 70° C for 15 minutes produced the 5-O-monoacetate [4-5], confirming the presence of the 5 hydroxyl with NMR analysis. Treatment of [4-4] with n-Bromosuccinimide produced the 5-O ketone, further confirming the absence of a free C-13 hydroxyl on the basis of NMR and UV spectral data.



[4-4] - Iodine/Silver Acetate Product



[4-5] - Acetate

Figure 4-4 : Iodine/Silver Acetate Product [4-4] and Acetate

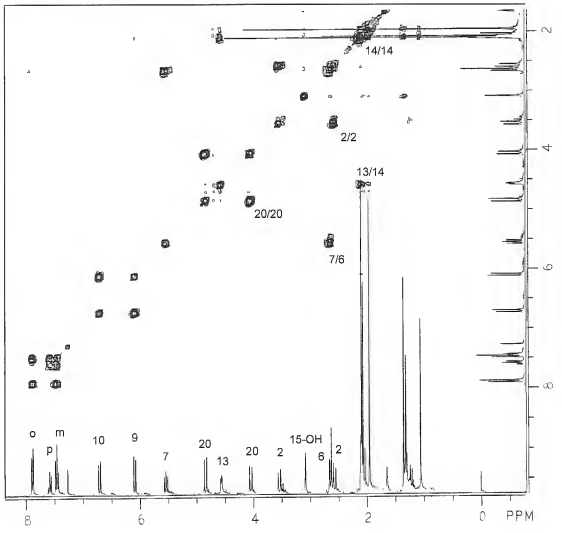


Figure 4-5 : H,H-COSY Spectrum of [4-4]

The ^{1}H NMR spectrum was similar to brevifoliol in most respects, with a few revealing differences. The AB quartet normally seen from H-2β became a clean doublet (J=13.8 Hz) deshielded from δ 2.36 to δ 3.31, and coupled to the H-2α proton, which was deshielded from δ 1.49 to δ 2.34. This deshielding and the coupling patterns indicated the loss of H-3β with the possible formation of a double bond between C-3 and C-4.

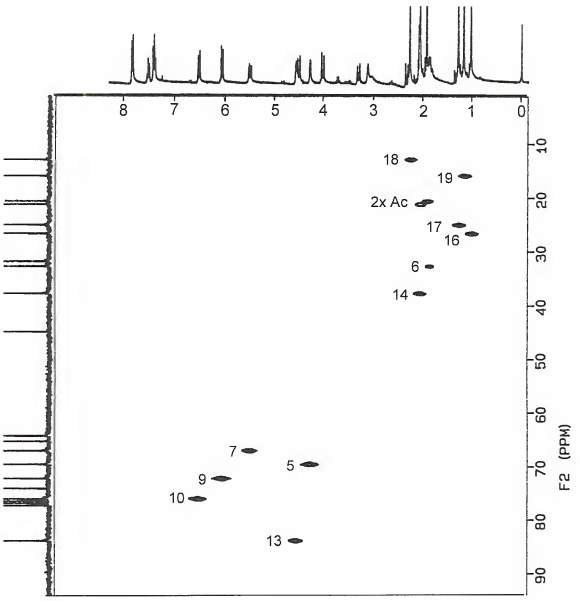


Figure 4-6 : HETCOR Spectrum of [4-4]

Recorded on Varian VXR 300 spectrometer at 75.432 MHz in $CDCl_3$ with TMS as internal standard. F_1: 3000.3 Hz; F_2: 15528 Hz; Acquisition time 65.9 sec; D_1: 1 sec; Ambient temperature; Decouple proton; Level 70 high power; PW: 90°; 128 repetitions X 128 increments; Waltz 16 modulation; pseudo echo; FT size: 2K X 512 data points; time: 5 hours.

Elemental analysis and FAB-MS gave the molecular formula $C_{31}H_{36}O_8$, which indicated essentially that loss of one molecule of water and dehydrogenation had taken

place. The presence of a hydroxyl was indicated by the fact that the compound would still undergo acetylation to form a monoacetate.

The C-15 hydroxyl was still present in both the carbon and proton spectra, and the only other significant change in the spectra occurred with the C-20 signals. The two broad singlets normally seen around 5 ppm were replaced by an isolated spin system of doublets at 4.35 and 3.92 which resemble the H-20 oxetane pattern seen in taxol.

Experimental

Brevifoliol Triacetate [3-5]

To a solution of brevifoliol (0.2 g) in acetic anhydride (2 ml) was added 0.2 ml of a 2% solution of boron trifluoride etherate in acetic anhydride to give a final concentration of 0.2% of boron trifluoride etherate. After 20 min at room temperature, the mixture was diluted with water. After another 10 min. the solid was separated, washed, taken up in ether and concentrated to dryness. The solid was crystallized from ether in ligroin to give [3-5] as a colorless crystalline solid, yield, 0.2 g; m.p. 214-216 ° C.

Oxidation with Jones Reagent to [4-1]

A solution of brevifoliol (0.2 g) in acetone (10 ml) was treated with Jones reagent (2 ml) added in small portions and with stirring. Initially TLC analysis of the reaction mixture showed that a yellow color giving spot appeared above that of the starting material. Gradually the first product changed into an even faster moving component. When this latter was the predominant product, the reaction was stopped by the addition of water and extraction with chloroform. After concentration of the solvent, the product was chromatographed on a silica column in 1:1 chloroform/ ligroin. Elution with chloroform gave the major component, which was obtained as a colorless crystalline solid, yield, 0.05 g; m.p. 234-236 ° C.

Action of Boron Trifluoride on Brevifoliol [4-3]

Brevifoliol (0.3 g) was dissolved in acetone (10 ml) and to the solution was added 1 ml of 1% boron trifluoride etherate in acetone to make a 0.1% overall concentration of boron trifluoride in the reaction mixture. After 3 h, water was added, the solid filtered and after drying, subjected to chromatography on silica gel in chloroform/ ligroin (1:1). The major band obtained with the same solvent was crystallized from ether/ligroin, yield, 0.1 g, m.p. 162-165 ° C.

^{1}H NMR ($CDCl_3$, Varian Unity 600 MHz, δ): 1.16, s (H-19); 1.03, s (H-16); 2.08 (cm, H-14α); 1.27, s (H-17); 2.32 (d, J=13.8 Hz, H-2α); 1.92 (s, methyl, 9-acetate); 1.84 (m, H-6α); 1.98 (m, H-6β); 2.26 (s, H-18); 2.05 (s, 7-acetate methyl); 3.30 (d, J=13.8 Hz, H-2β); 2.28 (cm, H-14β); 2.95, br s (C-15 OH, exchangeable with D_2O); 4.38 (t, J=7.2 Hz, H-13β); 4.28 (br s, H-5β); 4.54 (d, J=13.2 Hz, H-20 B); 4.54 (d, J=13.2 Hz, H-20 B); 5.51 (dd, J=4.8, 11.4 Hz, H-7α); 6.07 (d, J=10.1 Hz, H-9α); 6.54 (d, J=10.1 Hz, H-10β); 7.45 (t, J=7.5 Hz, H-Bz-*meta*); 7.57 (t, J=7.5 Hz, H-Bz-*para*); 7.89 (d, J=7.5 Hz, H-Bz-*ortho*).

^{13}C NMR ($CDCl_3$, Varian VXR 300 MHz, δ): 11.4 (C-18 methyl, q); 13.4 (C-19 methyl, q); 20.6 (7-O acetate methyl, q); 21.1 (9-O acetate methyl, q); 28.0 (C-2, t); 34.1 (C-6, t); 39.4 (C-3, d); 44.6 (C-14, t); 46.6 (C-8, s); 67.5 (C-10, d); 70.6 (C-7, d); 72.6 (C-5, d); 73.2 (C-9, d); 112.2 (C-20, t); 124.0 (C-13, d); 128.4 (C-Bz-*ortho*, d); 129.6 (C-Bz-*meta,* d); 130.2 (C-Bz-*ipso*, s); 132.9 (C-Bz-*para*, d); 134.2 (C-12, s); 145.9 (C-1, s); 146.4 (C-11, s); 150.4 (C-4, s); 165.2 (CO-Ph, s); 169.9 (CO-Acetate, s); 170.4 (CO-Acetate, s).

Reaction with Iodine and Silver Acetate [4-4]

To a solution of brevifoliol (0.5 g) in benzene (15 ml) were added iodine (0.7 g) and silver acetate (0.75 g) and the mixture stirred at room temperature for 20 h. TLC

showed that the starting material was absent and was replaced by two faster moving components. The mixture was filtered and the filtrate washed successively with aqueous sodium bisulfite and water and concentrated to dryness. Chromatography on silica gel in 4:1 chloroform/ligroin gave the major band, which was obtained as a colorless crystalline solid, total yield, 0.12 g; m.p. 250-252 ° C.

^{1}H NMR ($CDCl_3$, Varian Unity 600 MHz, δ): 1.16, s (H-19); 1.03, s (H-16); 2.08 (cm, H-14α); 1.27, s (H-17); 2.32 (d, J=13.8 Hz, H-2α); 1.92 (s, methyl, 9-acetate); 1.84 (m, H-6α); 1.98 (m, H-6β); 2.26 (s, H-18); 2.05 (s, 7-acetate methyl); 3.30 (d, J=13.8 Hz, H-2β); 2.28 (cm, H-14β); 2.95, br s (C-15 OH, exchangeable with D_2O); 4.38 (t, J=7.2 Hz, H-13β); 4.28 (br s, H-5β); 4.54 (d, J=13.2 Hz, H-20 B); 4.54 (d, J=13.2 Hz, H-20 B); 5.51 (dd, J=4.8, 11.4 Hz, H-7α); 6.07 (d, J=10.1 Hz, H-9α); 6.54 (d, J=10.1 Hz, H-10β); 7.45 (t, J=7.5 Hz, H-Bz-*meta*); 7.57 (t, J=7.5 Hz, H-Bz-*para*); 7.89 (d, J=7.5 Hz, H-Bz-*ortho*).

^{13}C NMR ($CDCl_3$, Varian VXR 300 MHz, δ): 13.1 (C-18 methyl, q); 16.1 (C-19 methyl, q); 20.8 (7-O acetate methyl, q); 21.5 (9-O acetate methyl, q); 25.3 (C-17 methyl, q); 26.9 (C-16 methyl, q); 31.7 (C-2, t); 38.2 (C-6, t); 142.7(C-3, d); 45.2 (C-8, s); 38.6 (C-14, t); 65.6 (C-1, s); 67.2 (C-7, d); 76.2 (C-10, d); 69.8 (C-5, d); 74.3 (C-15, s); 84.0 (C-13, d); 72.4 (C-9, d); 64.4 (C-20, t); 128.8 (C-Bz-*meta,* d); 129.2 (C-Bz-*ipso*, s); 129.4(C-Bz-*ortho*, d); 133.4 (C-Bz-*para*, d); 135.5 (C-12, s); 142.7 (C-4, s); 146.4 (C-11, s); 164.5 (CO-Ph, s); 169.4 (CO-Acetate, s); 170.3 (CO-Acetate, s).

FAB-MS (dithiothreotol/dithioerythrotol / TFA, m/z): 577 [M+Na]; 537 [M^+ NaOH]; 433 [M^+-$NaO_2C_7H_5$]; 373 [M^+-$NaO_2C_7H_5$ -HOAc]; 313 [M^+-$NaO_2C_7H_5$ – 2x HOAc]; 253 [M^+-$NaO_2C_7H_5$ –3x HOAc].

CI-MS (methane, m/z): 537.9 [MH^+ -H_2O]; 373.6 [MH^+ -H_2O–HOAc– C_6H_5COOH].

Acetylation of [4-4] to [4-5]

A sample of [4-4] (.05 g) was acetylated in acetic anhydride (2 ml) and pyridine (0.5 ml) at room temperature for 20 h. After addition of water, the solid was filtered and crystallized from ether in ligroin, m.p. 250-254 ° C.

^{1}H NMR ($CDCl_3$, Varian Unity 600 MHz, δ): 1.04 (s, H-16); 1.19 (s, H-19); 1.28 (s, H-17); 1.91 (s, methyl, 9-acetate); 1.77 (br dd, H-14α); 2.02 (m, H-6β); 2.05 (s, 7-acetate methyl); 2.27 (s, H-18); 2.19 (s, 5-acetate methyl); 2.22 (cm, H-14β); 2.34 (d, J=13.8 Hz, H-2α); 3.01, (br s, C-15 OH, exchangeable with D_2O); 3.31 (d, J=13.8 Hz, H-2β); 3.90 (d, J=13.2 Hz, H-20 B); 4.35 (d, J=13.2 Hz, H-20 A); 4.50 (br m, H-13β); 5.41 (dd, J=3.6, 13.2 Hz, H-7α); 5.46 (d, J=4.2 Hz, H-5β); 6.04 (d, J=10.1 Hz, H-9α); 6.57 (d, J=10.1 Hz, H-10β); 7.45 (t, J=7.5 Hz, H-Bz-*meta*); 7.57 (t, J=7.5 Hz, H-Bz-*para*); 7.89 (d, J=7.5 Hz, H-Bz-*ortho*).

^{13}C NMR ($CDCl_3$, Varian VXR 300 MHz, δ): 12.9 (C-18 methyl, q); 16.1 (C-19 methyl, q); 21.7 (7-O acetate methyl, q); 21.5 (9-O acetate methyl, q); 21.7 (5-O acetate methyl, q); 25.3 (C-16 methyl, q); 27.0 (C-17 methyl, q); 31.5 (C-2, t); 32.9 (C-6, t); 38.3 (C-14, t); 45.2 (C-8, s); 63.5 (C-1, s); 65.4 (C-20, t); 67.6 (C-7, d); 70.3 (C-5, d); 72.3 (C-9, d); 74.4 (C-15, s); 76.3 (C-10, d); 83.2 (C-13, d); 127.3 (C-4, s); 128.8 (C-Bz-*meta,* d); 129.1 (C-Bz-*ipso*, s); 129.4(C-Bz-*ortho*, d); 133.4 (C-Bz-*para*, d); 135.5 (C-12, s); 142.7(C-3, d); 145.6 (C-3, s); 147.6 (C-11, s); 164.6 (CO-Ph, s); 169.6 (CO-Acetate, s); 170.3 (CO-Acetate, s); 170.8 (CO-Acetate, s).

Reaction with N-Bromosuccinimide and Silver Acetate [4-6]

A solution of brevifoliol (0.2 g) in benzene (10 ml) was stirred with N-bromosuccinimide {NBS} (0.125 g, recrystallized from water). After 2 h the starting material was absent with two faster moving compounds being present. To the reaction mixture was added silver acetate (0.125 g) and stirred for another 2 h.

At this point, the previous major compound moved further to give a new product. The mixture was filtered, the filtrate washed with aqueous sodium bisulfite, followed by water and concentrated to dryness. The product was chromatographed on a silica column in 4:1 chloroform/ ligroin. The major component was obtained as a colorless crystalline solid, yield, 0.1 g. The compound was found to be identical with the product obtained from the reaction of brevifoliol with iodine and silver acetate.

Reaction of [4-4] with N-Bromosuccinimide [4-7]

A solution of [4-4] (0.04 g) in benzene (5 ml) was stirred with N-bromosuccinimide (25 mg) at room temperature. After 2 h, TLC showed formation of a slightly faster moving compound, which was separable from the starting material only after 2 or 3 developments of the TLC plate. The reaction mixture was washed with aqueous sodium bisulfite, followed by water and concentrated to dryness. The product was crystallized from ether in ligroin, m.p. 185-188 °C.

CHAPTER 5
TAXANE CONSTITUENTS OF THE NEEDLES OF *TAXUS* X *MEDIA*

As discussed in Chapter 3, the yield of taxol from the bark of *Taxus brevifolia* by using the conventional methods of isolation was of the order of 0.01%. It was also shown that through the use of these same methods, no other useful analogues could be isolated in any significant yields. As a consequence of these results and strong ecological considerations, an intense search was started with the aim of finding a source that is renewable, and which can match the bark in the yield of taxol. Many of the available species of *Taxus*, as well as the various parts of these plants were examined through the use of analytical high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). These searches led to the selection of the needles of the ornamental yew, *Taxus* x *media* Hicksii as a possible answer to the problem. The ornamental yew is capable of being grown in a nursery type setting, and on a large scale, so that the needles may be clipped twice a year, and the taxol, which is found to be present to the extent of 0.01% be isolated from them.

At the time of this research (1992-93), almost all of the studies carried out on this species consisted of HPLC analyses. Other than the isolation of taxol by the standard procedure with a total yield of 0.006%, no information had been published either on the taxane constituents, or even a method for the practical isolation of them. In these HPLC analyses, it was recognized that in the extracts of the ornamental yew, taxol was accompanied by other co-eluting taxanes and these could contribute some errors in the total yield calculations. These co-eluting taxanes were isolated in minute yields, in the form of two components (0.8 mg and 1.2 mg); each representing an equilibrium mixture

of two components. On the basis of NMR spectral evidence, structures were assigned to these two components (Castor & Tyler, 1993).

Due to the presence of pigments, waxes and other impurities, the isolation of taxol and other taxanes from the needles was expected to be more difficult when compared to their isolation from the bark of *T. brevifolia*. A project was started in this laboratory to meet the need for a practical method for the isolation of taxol and other related taxanes from the bark and needles of various *Taxus* species in spite of these challenges. The application of a preparative scale reverse phase column chromatography technique proved to be surprisingly successful in the processing of the extracts of *T. brevifolia*.

To begin with, the HPLC analysis of the extract of the needles of the ornamental yew, as shown in Figure 5-2, clearly shows that taxol is accompanied by several major taxane components, which are present in much higher concentrations than taxol itself. In view of such relatively high concentrations of these components, it is surprising that only such minute amounts of two of these mixtures could be isolated earlier, as indicated above. Also, no other characterizing data were provided other than the spectral data. This laboratory's objective was the development of a simpler procedure for the isolation of taxol with potential for large-scale use, in addition to more fully characterizing the major taxanes present in the extract. The needles of the ornamental yew (200 lbs., dried) were received through the courtesy of Hauser Company, Boulder, CO, during May-June 1993.

The extraction was carried out three or four times using methanol and the extract concentrated to a syrup. The resulting concentrate was then partitioned between water and chloroform, and the organic layer containing the taxane fractions was concentrated to a thick semi-solid mass, which was used directly in the next step.

The reverse phase column procedure was carried out similar to what was used with the needle extract of *T. brevifolia*, as described in Chapter 3. Approximately 200 g. of the chloroform extract was dissolved in acetonitrile (see experimental) and stirred with the equilibrated C-18 bonded silica. This slurry was then diluted to the appropriate concentration of the acetonitrile and the added to the column prepared from 800 g of the C-18 silica. Elution was carried out using a step gradient: 30, 35, 40, 45, 50 and 60% acetonitrile in water, and the eluate collected in fractions of 200 ml. As was seen in the case of the columns on the bark extract of *T. brevifolia*, when the fractions remained at room temperature for about a week, crystals began to separate from the fractions in different regions of the elution. These were filtered and further purified by either recrystallization or a small column of normal phase silica where necessary.

The progress of elution of the column is shown in Figure 5-3. As anticipated, taxol was accompanied by two other taxanes, which were present in higher concentrations than taxol. However, all of these crystallized out of the fractions.

The early fractions contained the bulk of the UV absorbance, and from these could be isolated a crystalline solid, which was a non-taxane compound. The next major component that emerged with the 35-40% acetonitrile in water was shown to be brevifoliol as described in Chapter 3. With the 45-50% acetonitrile and water solvent were eluted taxane I, taxane II, followed by taxol, all of which crystallized from their respective fractions, with some overlap.

The column was finally washed with a mixture of methanol and ethyl acetate/ligroin (2:1:1) which stripped the column of all the waxes, chlorophylls and other pigments. After, washing with methanol, followed by 25% acetonitrile and water the column was made ready for another run. Figure 5-1 shows the steps involved in the fractionation of the extract of *Taxus* x *media* Hicksii.

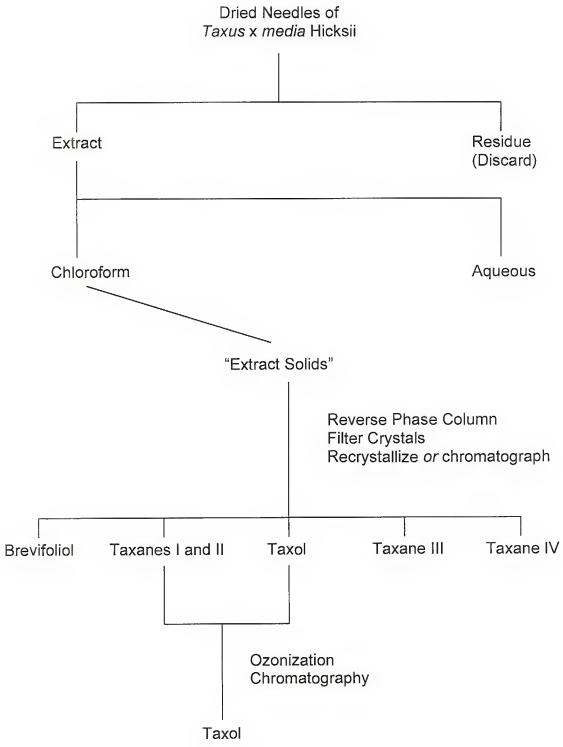


Figure 5-1 : Fractionation of the Extract of *Taxus* x *media* Hicksii Needles

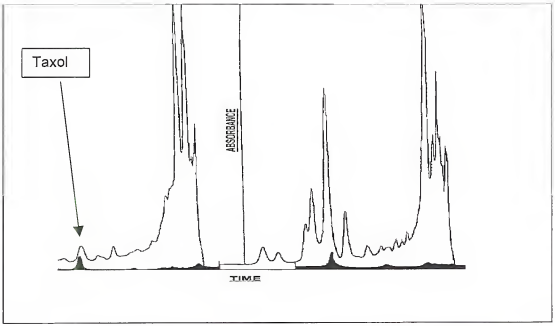


Figure 5-2 : HPLC Trace of Taxanes Coeluting with Taxol.

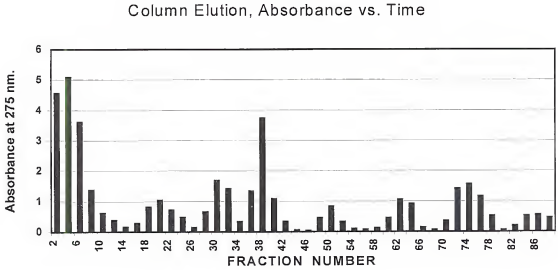


Figure 5-3 : Progress of Elution of Taxanes from Reverse Phase Column.

The reverse phase column run on 600 g of the extract obtained from 12 Kg of the dried needles was applied to a column prepared from 3 Kg of the C-18 bonded silica. The yields of the products obtained were good and important values are listed below.

Brevifoliol [3-1]

The fractions containing this component were combined, concentrated to dryness and chromatographed on a normal phase silica column. The major component was obtained as a colorless crystalline solid, which was found to be identical with brevifoliol [3-1] on the basis of its spectral data.

Taxanes I [5-1] and II [5-2]

Additional chromatography of the mixture of the taxanes I and II and taxol on a normal phase silica column gave some separation of the two taxanes. Although they could be further separated and obtained as crystalline solids, they still represented an equilibrium mixture, as was indicated by the ^{1}H- and ^{13}C NMR spectra of the individual crystalline samples. From the spectral data, these two were identified as a mixture of 5-O-cinnamoyl-10-acetyl taxicin I [5-1] and 5-O-cinnamoyl-9-acetyl taxicin I [5-2], which were isolated (Chmurney *et al.* 1993) from the needles of *T. x media* Hicksii and from the needles of *T. baccata* (Appendino *et al.* 1992). The former authors obtained them in quantities not sufficient for physical properties, and the latter authors obtained them as amorphous powders, by using HPLC and preparative TLC.

The mixture of [5-1] and [5-2] on acetylation with acetic anhydride and pyridine gave the triacetate [2-1], which was obtained as a crystalline solid and was found to be a single entity unlike the starting material. It was also identical with the taxane III (see below).

Taxane III [2-1]

The crude crystalline solid obtained from the reverse phase column was recrystallized. Its spectral and analytical data agreed with those given for 5-O-cinnamoyl-2α,9α, 10β-triacetyl taxicin I (Appendino *et al.* 1993; Baxter *et al.* 1962).

Taxane IV [2-2]

This was also purified by recrystallization of the crude crystals obtained directly from the fractions. It was found to be identical with 5-O-cinnamoyl 2α, 9α, 10β-triacetyl taxicin II, described by Appendino *et al.* (1992) and Baxter *et al* (1962).

Taxol [5-3]

The chromatography using normal phase silica column as described under taxanes I and II yielded taxol, which was purified by crystallization. The sample was still contaminated with some of the taxanes I and II. For complete purification, the mixture was subjected to ozonolysis which converted these two taxanes to more polar compounds from which taxol could be readily separated and obtained pure. Using this method, taxol was obtained in a yield of 0.015% based on the dry needles. This was significantly better than the reported yield of 0.006% (Witherup *et al.* 1990).

Ozonolysis of [2-2]

Because of the presence of the cinnamoyl ester function in compounds [5-1], [5-2], [2-1] and [2-2], they all undergo ozonolysis. This method gives a convenient way of separating taxanes [5-1] and [5-2] from taxol, with which they co-elute. In order to determine the nature of the product of ozonolysis, taxane [2-2] was subjected to this reaction and the product recovered and obtained as a crystalline solid. Its NMR spectral characteristics indicated a hydrated aldehyde with the structure shown in [5-6].

Thus, in summary, dried needles of *Taxus* x *media* Hicksii were extracted and the total chloroform extract applied to a C-18 reverse phase column. A number of components were separated, such as brevifoliol, taxanes I-IV and taxol, which crystallized out directly from the fractions. Separation of taxol from taxanes I and II could be carried out directly by ozonolysis of the mixture, followed by chromatography on either a normal phase or reverse phase silica column.

Experimental

Extraction:

Dried needles of *Taxus x media* Hicksii (50 lbs) were extracted with methanol as described in Chapter 3. The combined concentrate was partitioned between water and chloroform (10 gallons each). The organic layer was separated and the extraction repeated twice more using 5 and 3 gallons of the solvent, respectively.

The combined chloroform layers were concentrated under reduced pressure to yield a dark green semi-solid, representing approximately 5% of the weight of the dried needles.

Chromatography:

Approximately 800 g of C-18 bonded silica gel was poured into a glass Michell-Miller type column (2.5 x 24") using methanol (Ace Glass, 1430 North West Blvd., Vineland, NJ 08360). The column was equilibrated with 25% acetonitrile in water. The chloroform extract solids (200 g) was dissolved in acetonitrile (400 ml) in a 4 L stainless steel beaker, by warming in a hot water bath. To this was added approximately 200 g equivalent of the equilibrated C-18 bonded silica (20-25% of the column material). While the mixture is being stirred vigorously, 25% acetonitrile and water 500 ml was added, followed by water (approximately 800 ml). After stirring for 15 min. it was checked for

uniformity of the slurry and the absence of oily or waxy material, or lumps. The slurry was filtered under gentle suction and the solid was resuspended in approximately 500 ml of the filtrate to give a thin enough slurry for pouring. It was added to the column, the container rinsed and the rinse transferred to the column.

The remainder of the filtrate was pumped onto the column using a metering pump (Pulsa 680, Pulsafeeder Inc., Rochester, NY). After the sample addition was completed, elution was started using 30% acetonitrile and water. This was followed by 35, 40, 45, 50 and 60% acetonitrile and water. The column was then washed with 100% methanol. Final washing of the column with a mixture of methanol and ethyl acetate and ligroin removed the green pigments and other lipid-soluble components.

Fractions of 200 ml volume were collected and monitored by UV absorbance, TLC and analytical HPLC. After this, those fractions that contained significant UV absorbance and/ or components detectable by TLC or HPLC were set aside for 7-10 days, whereby crystals began to appear from a number of fractions. These were filtered in groups, characterized and treated appropriately, as described below.

Characterization of the Taxane Components of *Taxus* x *Media* Hicksii

Brevifoliol [2-1]

Fractions from the 40% acetonitrile and water were concentrated to dryness, the solid taken up in chloroform and applied to a column of normal phase silica (40 g). Elution with 2-5% acetone in chloroform gave the major band. The fractions that contain this component were combined, concentrated to dryness and the solid crystallized from acetone in ligroin. The crystalline product, yield, 0.8 g (0.02%) m.p. 220-222 ° C was found to be identical on the basis of NMR spectral data with brevifoliol described in Chapter 3.

Taxanes I and II [5-1] and [5-2]

The crude crystals that separated out from the fractions (8 g) consisting of [5-1], [5-2] and taxol [5-3] were processed by two methods. In one, the mixture (4 g) was taken up in chloroform and ligroin (3:1, 50 ml) and applied to a column of normal phase silica (60 g). The mobile phase was successively changed to chloroform, 2% acetone, 5% acetone, 2% methanol and 5% methanol in chloroform. Compounds [5-1] and [5-2] appeared in the 2-5% acetone and chloroform eluate partially separating from each other. Continuing with 2% methanol in chloroform gave taxol with small amounts of [5-1] and [5-2].

To obtain further purification of [5-1] and [5-2] the mixture was taken up in 40% acetonitrile and water and applied to a column of C-18 bonded silica. The column was eluted with 45 and 50% acetonitrile and water. As the fractions from the 45% acetonitrile and water elution stood for about a week, crystals appeared over a range of tubes and these were filtered in groups. Although [5-1] and [5-2] were separated, such that each contained the other to the extent of 10% or less, recrystallization gave worse mixtures, thus suggesting that isomerization (or equilibration) was taking place during the process. Data obtained on a crystalline (9:1 mixture of [5-1] and [5-2]: m.p. 136-138° C, $[\alpha]_D^{23}$ +214° (c 1.04, $CHCl_3$); (lit. Appendino *et al.* 1992 on an amorphous sample, m.p. 163-165° C and $[\alpha]_D^{23}$ +185 °).

Analysis calculated for $C_{31}H_{38}O_8$, H_2O: C, 66.89; H, 7.24. Found: C, 66.51; H, 7.19.

The ^{1}H- and ^{13}C NMR spectra of the crystalline [5-1] and [5-2] gave evidence of mixtures of two compounds. From the spectral data, these two were identified as a mixture of 5-O-cinnamoyl-9-acetyl taxicin I [5-1] and 5-O-cinnamoyl-10-acetyl taxicin I [5-2] described by Chmurney *et al.* (1993) from *Taxus x media* Hicksii and by Appendino *et*

al. (1992) from *Taxus baccata*. The former authors isolated insufficient amounts for characterization and the latter authors obtained them as amorphous powders by using HPLC and preparative TLC.

The mixture of [5-1] and [5-2] on acetylation with acetic anhydride and pyridine gave the acetate, readily obtained as a crystalline solid, m.p. 238-241° C, the NMR spectrum of which showed that it was a single entity, unlike the starting material. It was also identical with taxane III (see below).

Taxane III [2-1]

The crude crystals of taxane III obtained from the fractions with 50% acetonitrile and water were filtered and recrystallized from acetone in ligroin to obtain colorless needles, yield, 0.8 g (0.02%); m.p. 238-241° C, $[\alpha]_D^{23}$ +214 ($CHCl_3$, c 1.04); (lit. +218, Baxter, 1962); FAB-MS (m/z): 645 (M^+ +Na); 623 (M^+ + H); 475 [$(MH)^+$ - 148 (cinnamoyl)], 415 (475-AcOH); 355 (415-AcOH); 295 (355 - AcOH). The spectral data showed that it is the 5-O-cinnamoyl-2α, 9α, 10β-triacetyl taxicin I (Appendino *et al.*1992; Baxter *et al.* 1962).

Analysis calculated for $C_{35}H_{42}O_{10}$, H_2O: C, 65.61; H, 6.92. Found: C, 66.00; H, 6.72.

Taxane IV [2-2]

This compound also crystallized out directly from the fractions. The crude crystals were purified by recrystallization from acetone and ligroin, yield, 0.8 g (0.02%); m.p. 265-267° C; $[\alpha]_D^{23}$ +133°(C 0.98, $CHCl_3$); (lit. +137, Baxter *et al.* 1962); FAB-MS: 607 (MH^+); 459 (607 - 148 (cinnamate)); 399 (459 - HOAc); 339 (399 - HOAc); 279 (339 - HOAc).

Analysis calculated for $C_{35}H_{42}O_9$: C, 69.02; H, 7.03. Found: C, 69.29, H, 6.98.

The analytical and spectral data of [2-2] indicated that it was identical with 5-O-cinnamoyl taxicin II: 2α,9α,10β-triacetate described by Appendino *et al.* (1992) and Baxter *et al.* (1962).

Taxol [5-3]

In the silica column described above under the purification of compounds [5-1] and [5-2], taxol (approximately 0.8 g) was eluted by 2-5% methanol in chloroform. A small portion was crystallized from acetone and ligroin to obtain colorless needles of taxol. The ^{1}H NMR spectrum showed that the compound still had appreciable quantities of compounds [5-1] and [5-2]. To remove these compounds completely, ozonization was carried out on the rest of the sample in chloroform and methanol (9:1, 30 ml) at -70° C for 10-15 min. The reaction mixture was treated with dimethyl sulfide (0.5 ml) and let stand at room temperature for 2 h.

After concentration to dryness, the sample was chromatographed on normal phase silica (25 g) in chloroform. Elution with 2% methanol in $CHCl_3$ gave taxol which was crystallized from ligroin to obtain pure taxol, free from compounds [5-1] and [5-2], yield, 0.5 g (0.012%). Its spectral properties agreed with those of an authentic sample.

Alternatively, the crude crystalline solid consisting of compounds [5-1] ,[5-2] and taxol was directly ozonized in chloroform and methanol as before (but without he intermediate silica column purification). After decomposition of the ozonide, and concentration, the sample was subjected to chromatography and taxol isolated from the column. It was crystallized as before to yield 0.75 g (0.015%). The products of ozonization of compounds [5-1] and [5-2] were more polar than the original compounds and separated from taxol in the normal phase silica column.

Ozonolysis of Compound [2-2]

A solution of compound [2-2] (1 g.) in chloroform and methanol (30 ml, 9:1) was cooled in a dry ice and acetone bath and saturated with ozone for 10-15 min. TLC showed that the starting material was absent and ozonide being formed (detected by spraying with starch and potassium iodide which gave a blue color). After the decomposition of the ozonide by dimethyl sulfide, the reaction mixture was washed with water and concentrated to dryness. The product was crystallized from acetone in ligroin to obtain colorless needles, yield, 0.8 g, m.p. 168-170° C, $[\alpha]_D^{23}$ +130 (c 1.06, pyridine); HRMS: 569.2239, Calc. for $C_{27}H_{38}O_{13}$, 569.2234.

CHAPTER 6
TAXANE CONSTITUENTS OF *TAXUS FLORIDANA*

Taxus floridana is a species of *Taxus*, native to Florida. Its distribution is said to be limited to a small area along the Apalachicola River. It is a shrub and used frequently as an ornamental plant. As it is so with the other species of *Taxus*, the leaves of *T. floridana* are also reputed to be toxic to livestock and humans.

During the intensive search to find alternative sources for taxol to replace the bark of the Pacific yew, many species of *Taxus* from the United States, Canada, Europe and Asia were examined. However, there was no study of the taxane constituents of *Taxus floridana*. Our laboratory undertook this task to evaluate its usefulness as a possible source for taxol.

There was also an impetus for this study from another source. In exploring alternative sources to replace the bark of the Pacific yew, the National Cancer Institute (NCI) was interested in knowing whether the *Taxus* plants can be grown under hydroponic conditions, as opposed to their growing in their natural state. If these plants can be so grown under hydroponic conditions, which will eliminate the problem of having to harvest the tree bark, the next question was whether they produce taxol in adequate yields. Accordingly, the NCI approached our laboratory, and that of Prof. George Hochmuth Jr. of IFAS, University of Florida, to study this aspect. The hydroponic cultural techniques were studied by the IFAS laboratory and the isolation and characterization of taxanes by our laboratory. It was soon found that the two most well-known species of *Taxus*, namely *T. brevifolia* and *T. baccata* could not be readily propagated under normal hydroponic conditions, because their growth rate was very

slow. However, *T. floridana* responded satisfactorily and could be propagated under available conditions. This species was therefore studied in our laboratory for its taxane constituents.

The needles of *T. floridana* were collected from the campus and were extracted without drying. After extraction with methanol as before, concentration to remove the solvent, and partition between chloroform and water, the organic layer was concentrated to a dark green semisolid. Fractionation was again carried out using the reverse phase column techniques as was described under the needles of the other *Taxus* species in Chapters 3 and 5.

The crude chloroform extract was first tested by analytical HPLC to see the elution pattern of the taxane constituents. Taxol was clearly recognizable at its normal location, and in contrast to the observation with the needles of *Taxus* x *media* Hicksii, where there were co-eluting taxanes, the taxol from the extract of the needles of *T. floridana* was relatively free from such interfering taxanes. There were other taxanes situated at different locations.

Elution of the reverse phase column was carried out using a step gradient of 30, 35, 40, 45, 50 and 60% acetonitrile/ water. When the fractions were let stand at room temperature for 3-5 days, taxol and several other taxanes crystallized out as before.

The initial eluates from the column from 25-30% acetonitrile/ water contained highly polar phenolic constituents. The first taxane component to appear from the reverse phase column emerged with the 30-35% acetonitrile/water solvent, and crystallized almost immediately. This was found to be 10-deacetyl baccatin III [2-7]. The next taxane was eluted with the 40% acetonitrile/ water and it was found to be identical with brevifoliol [3-1]. With the 45% acetonirile/ water, was eluted another crystalline compound which was found to be a new compound, and was named taxiflorine [6-1]. Continued elution with 50% acetonitrile/ water gave two crystalline compounds in

succession. One of these was identified as baccatin VI [6-2], and the second one was taxol [5-3].

Taxiflorine

Taxiflorine [6-1] was readily obtained as a colorless crystalline solid. Its elemental analysis agreed with the molecular formula $C_{35}H_{44}O_{13}$. Its ^1H NMR spectrum in $CDCl_3$ showed broad and rounded peaks with poor resolution. In DMSO-d_6, the spectrum gave sharper signals but showed double the number of peaks in certain positions. The ^{13}C spectrum also exhibited extra peaks, which suggested that the compound was a mixture of rotamers in equilibrium. One could infer the presence of ester functions from the spectra, with four acetates and one benzoate, and an oxetane ring.

Acetylation of taxiflorine gave a monoacetate [6-3], which gave sharp signals in its ^1H NMR spectrum, with the expected number of peaks, thus showing that it is a single compound, unlike the starting material. Although the acetate was isomeric with baccatin VI, it was different. The most striking difference between the two spectra was seen with the signal for the H-13. In the acetate of taxiflorine, this signal was at δ 5.60, while the same was found at δ 6.3 in baccatin VI. A comparison with other related taxanes showed that in those with the 6-membered A-ring, the H-13 signal appears at δ 6.2-6.5, whereas in taxanes with a 5-membered A-ring, as in the 11(15→1)-abeotaxanes, it appears at δ 5.4-5.7 (Appendino *et al.* 1993B).

Positions 9 and 10 in taxiflorine carry a free hydroxyl and a benzoate function. To locate the benzoate, a comparison of the signals due to H-9 and H-10 in taxiflorine were compared with the corresponding signals in the monoacetate. With the two signals at δ 6.30 and δ 5.90 in taxiflorine, the latter undergoes a down-field shift from δ 5.90 to

δ 6-20, whereas the peak at δ 6.30 remains essentially unchanged (δ 6.37). With the reasoning that the allylic H-10 must be more down-field than H-9, the signal at δ 6.30 may be assigned to H-9 and the one at δ 5.90 for the H-10. This leads to the assignment of the structure of taxiflorine as [6-1], with the hydroxyl at 9 and the benzoate at 10. Based on the $^{1}H,^{1}H$-COSY and $^{1}H,^{13}C$-HETCOR spectral data, the four acetate functions were assigned as 2α, 4α, 7β and 13α.

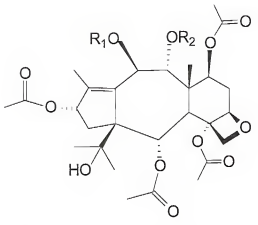
Benzoylation of [6-1] was carried out to yield the monobenzoate [6-4], which also gave a ^{1}H NMR spectrum that indicated that it was a single entity. Taxiflorine was also saponified to the heptaol and re-acetylated to the hexa-acetate [6-5]. The ^{1}H NMR spectra of [6-3], [6-4] and [6-5] are shown in Table 6-1.

The structure of [6-1] with the hydroxyl at C-10 and benzoate at C-9 has the potential for intramolecular transesterification occurring between the 9-benzoate, as well as the 7-acetate (Lewis *et al.* 1993). The fact that the monoacetate and the monobenzoate gave single products discounted the possibility that transesterification was responsible for the anomalous NMR spectra of [6-1]. To verify if the appearance of the spectrum is due to an rotameric equilibrium, the spectrum was taken in DMSO-d_6 at temperatures between -20° and 100°. At lower temperatures, the spectra were sharper and showed two sets of peaks for some protons. At higher temperatures, the peaks coalesced into a single set, as well as became broad to the extent that they were barely seen. This behavior suggested that the conformational equilibrium between the rotamers is responsible and that the presence of the 10-OH facilitates this process. As indicated earlier, during the exploration of possible alternative sources for taxol to replace the bark of the Pacific yew, scant, if any attention was paid to Taxus floridana. The species *T.* x *media* Hicksii (the ornamental yew) was selected as a possible source for taxanes in the future.

Table 6-1 : Proton NMR Spectra of Compounds [6-3], [6-4] and [6-5].

H #	Compound [6-3]	Compound [6-4]	Compound [6-5]
2	6.19, d, J=7.8 Hz	6.26, d, J=7.8 Hz	6.07, d, J=7.8 Hz
3	2.99, d, J=7.8 Hz	3.06, d, J=7.8 Hz	2.92, d, J=7.8 Hz
5	4.98, d, J=7.5 Hz	5.01, d, J=7.5 Hz	4.98, d, J=7.5 Hz
6α	2.68, m	2.70, m	2.52, m
6β	1.84, m	1.84, m	1.84, m
7	5.52, m	5.64, m	5.49, t, J=7.8 Hz
9	6.32, d, J=10.8HZ	6.48, d, J=10.8Hz	6.04, d, J=10.8Hz
10	6.37, d, J=10.8 Hz	6.72, d, J=10.8 Hz	6.27, d, J=10.8, Hz
13	5.62, t, J=7.8Hz	5.64, m	5.61, t, J=7.8Hz
14α	2.30, dd,J=7.4,14.2Hz	2.34, dd,	2.30, m
14β	1.72, dd,J=7.4,14.2Hz	1.78, m	1.72, m
16	1.16, s	1.24, s	1.15, s
17	1.19, s	1.21, s	1.13, s
18	1.72, s	1.72, s	1.83, s
19	1.64, s	1.95, s	1.66, s
20	4.50, 4.42, d, J=7.9 Hz	4.52, 4.44, d, J=7.2	4.47,4.38,d,J=7.5
Ph(2',6')	7.93, d	Unresolved	----
Ph(3',5')	7.45, t	7.24, mm	----
Ph(4')	7.62, t	7.37, m	----
Ph(2",6")	----	7.63, d, J=7.2Hz	----
Ph(3",5")	----	7.24, m	----
Ph(4")	----	7.37, m	----
OAc Me	2.02,s	2.14, s	2.11,s
OAc Me	2.14, s, (2x)	2.05, s	2.10, s
OAc Me	1.86, s	----	2.08, s
OAc Me	1.80, s	----	2.03, s
OAc Me	----	----	2.01, s
OAc Me	----	----	1.95, s

^{1}H NMR were recorded at 600 MHz in $CDCl_3$ on a Varian Unity 600 spectrometer at ambient temperature. Chemical shifts δ (ppm) are reported relative to TMS as an internal standard.

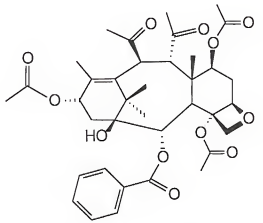


[6-1] - Taxiflorine $R_1 = H$ $R_2 = C_6H_5CO$

[6-3] - Taxiflorine Acetate $R_1 = CH_3CO$ $R_2 = C_6H_5CO$

[6-4] - Taxiflorine Benzoate $R_1 = R_2 = C_6H_5CO$

[6-5] - Hexa-Acetate $R_1 = R_2 = CH_3CO$



[6-2] - Baccatin VI

Figure 6-1 : Taxanes and Analogues from *Taxus* x *media* Hicksii

From the present studies, which compared the taxane composition of both species, it became clear that *T. floridana* would have been a much better choice, for the reasons given below:

1. Taxol is isolated more easily from the Florida yew than from the ornamental yew, because, in the former there are no co-eluting taxanes to interfere and which have to be removed as an additional step. The yields of taxol (0.01% from fresh leaves) are accordingly better with the Florida yew, than from that of the ornamental yew (0.015% from dried leaves).
2. Besides taxol, the Florida yew gives relatively high yields (0.05-0.06% from fresh leaves) of 10-deacetyl baccatin III, which is the most commonly used intermediate for the semi-synthesis of taxol. This compound is present in the ornamental yew in exceedingly low concentrations.
3. The other components that contain the oxetane ring, such as baccatin VI and taxiflorine, are also useful compounds for the synthesis of taxol analogues. In contrast, the taxanes from the ornamental yew that are 11,4/20 diene taxanes have no currently documented use.

The work described here is the first definitive investigation of the taxane constituents of Taxus floridana on a preparative scale giving the actual recovered yields of the crystalline compounds.

Experimental

Extraction

The needles and small twigs of Taxus floridana were collected from several bushes growing at different locations of the campus of the University of Florida. Likewise, they were made available from the plants growing under hydroponic

conditions. For convenience, they were extracted in fresh state. Several batches were collected from the plants on the campus ranging from 1-20 Kg. With the hydroponically grown plants, the smallest amount was 54 g of the fresh needles, and the largest, 2.5 Kg. The method of extraction, concentration, partition between water and chloroform were the same as was described in Chapter 3. The yield of the chloroform extract varied from 20-25 g per Kg of the fresh leaves and twigs.

Before the conditions for the reverse phase column chromatography were fully developed, an alternative procedure was tested for the purpose of making the sample preparation easier. The chloroform extract, especially of the needles, usually contains a higher amount of waxes, chlorophylls and other lipid-soluble components and can potentially pose problems in the preparation of the sample for applying to the column. For this reason, a study was made to see at what concentration of methanol or acetonitrile would be needed to obtain an essentially clear solution that can be applied to the column.

It was found that at least an 80% methanol in water would be necessary to prepare a 10% solution of the chloroform extract solids. However, at this concentration of the solvent, taxol and most of the other taxanes do not remain on the column. It was also found that if such a solution is passed through a column of C-18 bonded silica, almost all of the chlorophylls, waxes etc., remain on the column, while taxol and other taxanes appear in the effluent and washes. There was also a reduction of the solids content by 50-60%, which meant that the waxes and other lipid-soluble components account for this much of the chloroform extract and can be readily removed from the sample.

The material obtained by concentrating the effluent and washes was much less difficult to prepare as the slurried sample for applying to the column. In order to carry out this wax-removal operation, it was only necessary to use a ratio of 3 g of the C-18

silica for 1 g of the extract. The chlorophylls and waxes that were held up on the column could be readily removed by washing with a mixture or methanol/ ethyl acetate/ ligroin (2:1:1). Examination by TLC showed that this wash did not contain any taxane constituents. This procedure was not used in later trials, as methods were found for a successful and convenient preparation of the sample slurry made it unnecessary, as described in Chapter 3.

Characterization of the Taxane Constituents of *Taxus floridana*

The results given here represent the work carried out on a 20-Kg batch of the fresh needles.

10-Deacetyl Baccatin III [2-7]:

Elution with 30% acetonitrile in water gave this component which crystallized almost immediately. After a week, the crystals were filtered off, dried and recrystallized from methanol/ chloroform, yield, 12 g (0.06%); m.p. 232-234° C. The spectral properties were identical with those described in the literature (Chauviere *et al.* 1981, Appendino *et al.* 1993b).

Brevifoliol [3-1]:

Fractions from the 35-40% acetonitrile eluate, which contained this component but did not give a crystalline solid directly, were combined, concentrated to dryness and the solid (3 g) was applied to a normal phase silica column (120 g) in chloroform. Elution with 2% methanol in chloroform gave the major band, the fractions from which were combined, concentrated and the solid crystallized from acetone / ligroin to give 1 g of [3-1], m.p. 220-222° C. Its spectral data proved to be identical with those described in Chapter 3.

Taxiflorine [6-1]:

The crude crystalline solid (2.5 g) that separated from the fractions from 45% acetonitrile/ water was filtered and purified by recrystallization from acetone/ ligroin to give [6-1] as a colorless crystalline solid, yield, 1.2 g (0.006%); m.p. 254-255° C, $[\alpha]_D^{23}$ -26.1°.

Analysis calculated for $C_{35}H_{44}O_{13}$: C, 62.48; H, 6.59. Found: C, 62.12; H, 6.63.

Baccatin VI [6-2]:

Eluates from the 50% acetonitrile/ water gave crystals in a number of fractions. These were filtered into groups and tested by TLC and analytical HPLC. The earlier fractions contained mostly baccatin VI, with gradually increasing amounts of a slower compound, shown to be taxol. The crystals from the first group containing mostly [6-3] (3.5 g) were dissolved in chloroform (50 ml) and passed through a column of Florisil (20 g) for the purpose of decolorization. The effluents and washes were combined, concentrated to dryness and the solid crystallized from acetone/ ligroin to give pure [6-3] yield, 1.6 g. Together with the amount obtained from the next fraction, the total yield was 1.95 g (0.01%); m.p. 250-252 ° C (lit. 248-250 ° C decomp., Senilh *et al.* 1984); $[\alpha]_D^{23}$ -11° (chloroform, c 0.98) (lit. –5, chloroform, c 1.3, Senilh *et al.* 1984); MS(FAB); 737 $[M+Na]^+$, 697 $[M-H_2O]^+$.

^{1}H NMR ($CDCl_3$, 300 MHz, δ): 1.22 (17-Me, s); 1.60 (19-Me, s); 1.78 (16-Me, s); 1.87 (6α, cm); 1.99 (OAc-Me, s); 2.02 (OAc-Me, s); 2.04 (C-14β, unresolved mult.); 2.10 (18-Me, s); 2.10 (OAc-Me, s); 2.19 (OAc-Me, s); 2.20 (C-14α, unresolved mult.); 2.28 (OAc-Me, s); 2.50 (C-6β, cm); 3.18 (C-3, d, 6 Hz); 4.13 (C-20, d, 8.4 Hz); 4.34 (C-20, d, 8.4 Hz); 4.97 (C-5, d, 8.4 Hz); 5.55 (C-7, dd, 7.5, 9.3 Hz); 5.87 (C-2, d, 6.0 Hz); 5.99 (C-9, d, 11.1 Hz); 6.17 (C-13, dd, 7.6, 9.3 Hz); 6.22 (C-10, d, 11.1 Hz): 7.48 (Ar-meta, t, 7.8 Hz); 7.61 (Ar-para, t, 7.5 Hz); 8.09 (Ar-ortho, dd, 7.2, 1.3 Hz).

170.4 – AcCO
170.1 – AcCO
169.8 – AcCO
169.1 – AcCO
168.8 – AcCO
166.8 – BzCO
141.1 – 12
133.6 – Bz (p)
133.5 – 11
129.9 – Bz (m)
129.2 – Bz (q)
128.5 – Bz (o)

83.7 – 5
81.4 – 4
78.7 – 2
76.3 – 20
74.9 – 1
73.1 – 7
71.7 – 9
70.3 – 13
69.6 – 13
47.2 – 3
45.7 – 8
42.7 – 15

35.0 – 14*
34.4 – 6*
28.2 – 17 Me
22.6 – Ac Me
22.2 – 16 Me
21.3 – Ac Me
21.1 – Ac Me
20.8 – Ac Me
20.7 – Ac Me
14.9 – 18 Me
12.7 – 19 Me
* Interchangeable

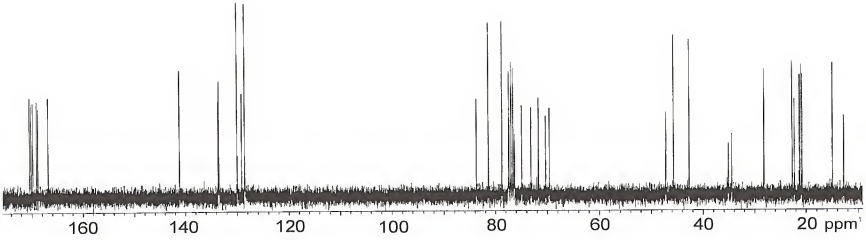


Figure 6-2 : Carbon NMR Spectrum of Baccatin VI

^{13}C NMR ($CDCl_3$, 300.075 MHz, δ): 75.0 (C-1); 78.8 (C-2); 47.5 (C-3); 81.6 (C-4); 83.8 (C-5); 34.3 (C-6); 73.3 (C-7); 45.8 (C-8); 71.9 (C-9); 69.7 (C-10); 133.5 (C-11); 142.0 (C-12); 70.4 (C-13); 35.3 (C-14); 42.8 (C-15); 22.4 (C-16); 28.3 (C-17); 12.9 (C-18); 15.0 (C-19); 76.5 (C-20); 20.7; 20.9, 21.2, 21.4, 22.7 (5x OAc Me); 168.6, 168.9, 169.6, 169.9, 170.2 (5X OAc CO); 166.8 (Bz CO); 129.2 (Bz ipso); 128.5 (Bz ortho); 129.9 (Bz meta); 133.5 (Bz para).

Analysis calculated for $C_{37}H_{46}O_{14}$: C, 62.18; H, 6.49. Found: C, 61.83; H, 6.45.

Taxol [5-3]:

Crystals (4.5 g) from the second part of the peak which contained mostly taxol. were combined dissolved in chloroform (60 ml) and chromatographed on Florisil(40 g). Elution with chloroform gave more of [6-3] and subsequent elution with 5% acetone in chloroform gave taxol, which was recovered by concentration of the appropriate fractions and crystallized from acetone and ligroin to obtain taxol, yield, 1.98 g (0.01%); m.p. 220-222 ° C. The spectral and chromatographic properties of the sample agreed with those of taxol.

Acetylation of Taxiflorine to [6-3]:

An aliquot of [6-1] (0.05 g) was acetylated by acetic anhydride (2 ml) and pyridine (0.5 ml) at room temperature for 16 h. Water was added, the solid filtered and purified by chromatography on a silica column using chloroform / ligroin (2:1) to obtain [6-3] as a white powder.

Benzoylation of Taxiflorine to [6-4]:

To a solution of [6-1] (0.05 g) in pyridine (2 ml) was added dropwise with stirring at 0-5° C, benzoyl chloride (0.1 ml). After 20 h water was added followed by 2N sulfuric acid and the solid filtered. The product was purified by chromatography as given under [6-3], to obtain [6-4] as a white powder.

Saponification and Acetylation of [6-1] to [6-5]:

A solution of taxiflorine (0.1 g) in methanol (5 ml) was treated with 2N potassium hydroxide (1 ml) and the mixture let stand at room temperature for 2 h. TLC showed that the starting material was no longer present, along with the appearance of a very slow moving component. The solution was acidified and extracted with chloroform (3x) and the combined extracts concentrated to dryness. The residue was dissolved in acetic anhydride (2 ml) and pyridine (0.5 ml) and let stand for 16 h. Addition of water, filtration of the solid and chromatography on a normal phase silica gave [6-5] as a white powder.

CHAPTER 7
NON-TAXANE COMPONENTS FROM THE BARK AND NEEDLE EXTRACTS

General

Some of the benefits of using reverse phase rather than normal phase chromatography have been described in previous chapters. Two important disadvantages of normal phase silica gel chromatography are the acidic nature of silica and the tendency for irreversible adsorption to occur. Both of these problems can lead to significant loss of the compound(s) of interest. Fortunately, almost all free acidic groups are capped during the bonding process used to make reverse phase silica, followed by a final capping with trimethylsilyl groups. This process effectively eliminates these problems of acidity and irreversible adsorption.

These properties allow recovery of many compounds that would normally not be amenable to silica gel chromatography. Glycosides, phenols, steroids and hydrophilic compounds are often difficult to chromatograph using normal phase silica gel. During the processing thousands of pounds of bark and lesser amounts of needles many interesting compounds were isolated. The taxane glycosides are most notable among these, especially now that the efficient removal of the glycosyl moiety has become possible (Rao, 1997). The relative abundance of taxane glycosides amenable to conversion into taxol gives further support to the use of reverse phase columns. Large amounts of valuable precursors are lost with the normal Polysciences isolation process (Boettner *et al.* 1979).

Flavonoids are chemicals generally found in plants that are ubiquitous and have been studied for hundreds of years. These compounds are generally yellow to red in color and are usually responsible for the colors seen in flowers and plants. Research into the possibility that flavonoids might possess useful biological activities has undergone a renaissance in recent years, after decades of neglect. Quercetin and its most common glycoside (rutin) are probably the most ubiquitous of the flavonoids, and have been used for many years to enhance immune response to pathogens. "Bioflavonoid complex" is mostly rutin and another flavonoid known as hesperidin, and can be purchased in most drug stores and herbal shops.

These flavonoid compounds were easily purified on the reverse phase columns. The future usefulness of flavonoids as bioactive compounds remains to be seen, but the evidence is growing. Some of these compounds have been reported to inhibit the reverse transcriptase enzyme of HIV virus *in vitro*, but further work is needed concerning the mechanism.

Insect molting hormones, commonly classed as "edcysones," are responsible for the maturation of larvae into adult form and have interested scientists for many decades. Originally, silk worms were extracted to obtain these compounds for research and in very low yields indeed. Efforts to find better sources for these compounds led to their discovery in some plants, including *Taxus baccata* (Hoffmeister 1966). Substantial amounts of β-ecdysterone, ponasterone A and other ecdysones were easily isolated during the workup of fractions from the reverse phase columns. All samples tested in this project had substantial amounts of these hormones, and were easy to isolate.

Usnic acid is a bright yellow compound which is known to grow in lichens. Due to its structure all signals in the proton NMR are singlets. It is commonly classed as a tricarbonyl and has peaks as high as 18.8, 13.3 and 11 ppm. An endophytic fungus of the pacific yew known as *Taxomyces andreanae* is capable of producing taxol in cell

culture (Stierle *et al.* 1993). It is a mystery how these two organisms that are so different are able to make a complex diterpene like taxol. In spite of this taxol producing fungus, it is most likely that the usnic acid was actually produced by lichens growing on the bark, and is not produced by the tree. Usnic acid is mentioned here because it was difficult to characterize and not uncommon in samples processed in this lab. Usnic acid is also known to be quite toxic and should be handled carefully.

Betuloside is a simple glycoside first isolated from the plant *Betula pendata* (Khan 1966). Animal studies using hepatotoxic agents indicate that betuloside has significant hepatoprotective activity. The mechanism by which this compound protects the liver is not known, but teas made from plants containing betuloside have been used in India for centuries for various problems. Betuloside is just one more example of the usefulness of preparative scale reverse phase chromatography.

Experimental

Analytical HPLC was performed using two different systems. For determinations of purity and quantitative information on composition, a setup with a Waters 600 E pump with gradient control, a Waters 996 photodiode array detector, and a Waters 717 autosampler, coupled with an NEC-386 computer and printer was used. Waters Millennium 1.1 program was used with the photodiode array system. For routine use, a combination of a Waters 501 pump with a U6K injector, a 486 tunable absorbance detector and a Goerz Servogor 120 recorder was used.

For analytical purposes, standard columns packed with C-8 bonded silica (Whatman Partisil®, 4.6 mm x 25 cm, 5μ) were used with either of the solvents: 50% acetonitrile in water, or a 5:4:1 mixture of acetonitrile, water and methanol.

For preparative scale purposes, stainless steel columns of two sizes were used: 4" x 4' and 6" x 6', fabricated by Fluitron Inc. (Ivyland, PA) and rated to 200 psi. The

columns were packed with C-18 or C-8 bonded silica gel (Spherisorb, 15-35 μ diameter, Phase Separations Inc., Norwalk, CT) as a slurry in methanol . After thorough washing with methanol, the columns were equilibrated with 25% acetonitrile in water.

Thin-layer chromatography was carried out using silica gel HF-60, 254+366 (EM Science/Fisher). Visualization was by a UV-lamp and by charring with 1 N H_2SO_4. Column chromatography was performed using silica gel (Fisher, 100-200 and 235-425 mesh) or Florisil (Fisher F-101, 100 mesh) with a solvent sequence consisting of ligroin/$CHCl_3$, $CHCl_3$, 2-5% acetone and finally, 2-10% MeOH in $CHCl_3$.

Melting points were determined on a Fisher-Johns hot stage apparatus and are uncorrected. The following instrumentation was used for the spectra recorded here: UV, Perkin Elmer λ3B; IR, Perkin Elmer PE-1420; and NMR, General Electric QE-300, Nicolet NY-300, Varian VXR-300, Varian Gemini-300 and Varian Unity-600 spectrometers. Mass spectra (FAB) were obtained on a Finnegan Mat 95Q spectrometer using a cesium gun operated at 15 KeV of energy.

Flavonoids

Quercetin Rutoside (Rutin)

3-[[6-O-(6-deoxy-α–L-mannopyranosyl)-β–D-glucopyranosyl]oxy]-2-(3',4'-dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one. m.p. : 186-189 °C (dec., turns brown at ~127 °C); $[\alpha]_D^{23}$ +14.06 (ethanol, c 1.02); $[\alpha]_D^{23}$ -39.76 (pyridine, c 1.06).

^{1}H NMR (DMSO-d_6, 300 MHz, δ): 12.6 (C-5 hydroxyl, br s, D_2O exchangeable); 7.57 (H-2', d, 2.2); 7.53 (H-6', dd, 8.6, 2.2); 6.86 (H-5', d, 8.3); 6.40 (H-8, br s); 6.21 (H-6, br s); 5.34 (H-1" glucosyl, br s); 4.42 (H-7" rhamnosyl, br s); 3.1-3.75 (CH-OH glycosyl, 6H, mult.); 1.03 (H-12" methyl, d, 6.0).

^{13}C NMR (DMSO-d_6, 300 MHz, δ): 177.5 (C-4, carbonyl); 164.1 (C-7, s); 161.4 (C-5, s); 156.9 (C-2, s); 156.7 (C-9, s); 148.5 (C-4', s); 144.8 (C-3',s); 133.5 (C-3, s); 121.9 (C-6', d); 121.4 (C-1',s); 116.4 (C-2',d); 115.4 (C-5',d); 104.2 (C-10, s) ;100.6 (C-1",d); 98.6 (C-6,d); 93.5 (C-8, d); 76.5; 76.0; 74.2; 71.9; 70.6; 70.55; 70.49; 70.1; 68.4; 67.2 (C-6", t); 18.0 (C-12" methyl, q).

IR, *v* max (KBr, cm^{-1}): 3340 (OH, bonded); 2920 (CH stretch); 1655 (C=O); 1620 (C=C); 1510 (aromatic); 1355 (C-O-C); 1290 (C-O-C); 1200 (C-O-C); 1055 (C-O-C); 970, 880, 810 (subst. aromatic); 730, 695.

Analysis calculated for $C_{27}H_{30}O_{16}$: C, 53.12; H, 4.95. Found: C, 52.88; H, 5.06.

Quercetin

2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one or 3,3',4',5,7-pentahydroxyflavone. Refluxed 300 mg of quercetin glycoside in 2 N H_2SO_4 for 3 hours, filtered, washed with water, yellow needles crystallized from aqueous ethanol, 150 mg (77%).

^1H NMR (DMSO-d_6, 300 MHz, δ): 12.5 (C-5 hydroxyl, br s, exchangeable with D_2O); 7.68 (H-2', d, J=2.2); 7.54 (H-6', dd, J= 2.0 and 8.4); 6.90 (H-5', d, J=8.4); 6.41 (H-8, d, J=2.0); 6.19 (H-6, d, J=2.4).

^{13}C NMR (DMSO-d_6, 300 MHz, δ): 175.7 (C-4, s); 163.8 (C-7, s); 160.7 (C-5, s); 156.9 (C-9, s); 147.6 (C-4', s); 146.9 (C-2, s); 145.0 (C-3', s); 122.0 (C-1', s); 120.0 (C-6', d); 115.6 (C-5', d); 115.3 (C-2', d); 103.0 (C-10, s); 98.2 (C-6, d); 93.4 (C-8, d).

Analysis calculated for $C_{15}H_{10}O_7$+ 2 H_2O: C, 53.26; H, 4.17. Found: C, 53.64; H, 4.04.

Sciadopitysin

This biflavonoid is a member of the amentoflavone group, m.p. : 302-304° C.

^{1}H NMR (DMSO-d_6, 300 MHz, δ): 13.05 (1H, s, 5'-OH); 12.90 (1H, s, 5-OH); 8.31 (1H, s, 7-OH); 8.17 (1H, dd, J=9.0, 2.4, 6'-H); 8.08 (1H, d, J=2.4, 2'-H); 7.60 (2H, d, J=9.0, 2''' H, 6''' H); 7.37 (1H, d, J=8.7, H-5'); 6.98 (1H, s. H-3''); 6.93 (2H, d, J=9, H-3''', H-5'''); 6.89 (1H, s); 6.78 (1H, d, J=2.4, H-8); 6.42 (1H, s, H-6''); 6.36 (1H, s, 6-H); 3.83 (3H, s); 3.80 (3H, s); 3.76 (3H, s).

^{13}C NMR (DMSO-d_6, 300 MHz, δ): 181.9; 181.8; 165.1; 163.5; 162.9; 162.1; 161.0; 160.5; 160.4; 157.2; 154.2; 130.8; 128.2; 127.7; 122.7; 122.3; 121.5; 114.4; 111.6; 104.1; 103.7; 103.55; 103.48; 103.1; 98.6; 98.0; 92.6; 55.9; 55.8; 55.4.

IR, *v* max (KBr, cm^{-1}) : 1660, 1650, 1620, 1610, 1570, 1510, 1430, 1370, 1240, 1180, 1160, 1050, 1030, 960, 910, 880, 830, 760.

Analysis calculated for $C_{33}H_{24}O_{10}$: C, 68.27; H, 4.17. Found: C, 67.94; H, 4.26.

β–Sitosterol-β–D-Glucoside

Identity of glycoside confirmed using authentic sample with mixed melting point, TLC, and IR spectrum, as well as ^{1}H- and ^{13}C NMR of the tetra-acetate, the aglycone, and the aglycone acetate. M.p. : 288-290 ° C (lit. varies from 280-300 ° C, 298 ° C Sucrow 1966). EI-MS: 414(2%) [M+ - glucosyl], 396(34%) [MH+ - glucosyl – H_2O]. CI-MS (methane): 413(9%) [MH+ - glucosyl – H_2], 397(100%) [MH+ - glucosyl – H_2O]. $[\alpha]_D^{23}$ -40.1 (pyridine, c 1.1); (lit. -41.0, c 1.33, pyridine, Swift 1952).

IR *v* max (KBr, cm^{-1}) : 3400 (-OH); 3090 (C=CH_2); 1650, 890.

Analysis calculated for $C_{35}H_{60}O_6$: C, 72.87; H, 10.48. Found: C, 72.48; H, 10.61.

β–Sitosterol-β–D-Glucoside Tetra-acetate

Acetylation of β–sitosterol-β–D-glucoside: Dissolved 500 mg in 5 ml acetic anhydride with 0.1 ml pyridine, then placed in hot water bath for 1 hour with stirring. After TLC indicated the reaction was complete the mixture was stirred with water for 15 minutes to decompose the anhydride, then extracted with chloroform 3 x at pH 4. Dried

over Na_2SO_4, concentrated, then crystallized from ethyl acetate in ligroin, yield 440 mg (68%); first crop.

M.p.: 165-167 ° C (lit. 171 ° C, Sucrow 1966). $[\alpha]_D^{23}$ –35.0 (pyridine, c 1.3); (lit. pyridine, c 1.33, -33.7, Swift 1952).

^{1}H NMR ($CDCl_3$, 300 MHz, δ): 5.37 (H-6, br d, J= 4.8); 5.21 (H-3', t, J=9.3); 5.08 (H-4', t, J=9.2); 4.95 (H-2', dd, J=9.3 and 8.1); 4.60 (H-1', d, J=8.1); 4.26 (H-6'b, dd, J=4.8 and 12); 4.11 (H-6'a, dd, J=2.4, 12); 3.70 (H-5', cm); 3.5 (H-3, cm); 2.07, 2.05, 2.02, 2.00 (4 X Ac CH_3, s); 1.0 (H_3C-19, s); 0.84 s; 0.82 s; 0.68 (H_3C-18, s).

^{13}C NMR ($CDCl_3$, 75 MHz, δ): 170.6, 170.3, 169.3, 169.2 (4 X Ac<u>C</u>=O, s); 140.3 (C-5, s); 122.1 (C-6, d); 99.6 (C-1', d); 80.0 (C-3, s); 72.9 (C-3', d); 71.7 (C-5, d); 71.5 (C-2', d); 68.5 (C-4', d); 62.1 (C-6', t); 56.7 (C-14, d); 56.0 (C-17, d); 50.1 (C-9, s); 45.8 (C-24, s); 42.3 (C-13, s); 39.7 (C-12, t); 38.9 (C-4, d); 37.2 (C-1, d); 36.7 (C-10, s); 36.1 (C-20, d); 33.9 (C-22, t); 31.9 (C-8, d); 31.8 (C-7, t); 29.4 (C-2, t); 29.1 (C-25, d); 28.2 (C-16, t); 26.1 (C-23, t); 24.3 (C-15, d); 23.0 (C-28, t); 21.0(C-11, d); 20.6, 20.5, 20.44, 20.42 (4 X AcMe); 19.7 (C-27, q); 19.3 (C-19, q); 19.0 (C-26, q); 18.7 (C-21, q); 11.8 (C-18, q); 11.8 (C-29, q).

IR, *v* max (KBr, cm^{-1}) : 1755, 1220, 1045, 910.

<u>β–Sitosterol</u>

M.p.: 138-140 ° C (lit. 137-138 ° C, Swift 1952); $[\alpha]_D^{23}$ -37.2 (chloroform, c 1.1); (lit. –38.2, chloroform, c 5.1, Swift 1952).

^{1}H NMR ($CDCl_3$, 300 MHz, δ): 5.36 (H-6, d); 3.50 (H-3, m); 2.30 m, 2.0 m, 1.0 s, 0.82 d, 0.7 s.

^{13}C NMR ($CDCl_3$, 75 MHz, δ): 140.7 (C-5, s); 121.6 (C-6, d); 71.8 (C-3, s); 56.7 (C-14, d); 56.0 (C-17, d); 50.1 (C-9, d); 45.8 (C-24, d); 42.3* (C-13, s); 43.2 (C-4, t); 39.7 (C-12, t); 37.2 (C-1, t); 36.4 (C-10, s); 36.1 (C-20, d); 33.9 (C-22, t); 31.9 (C-8, d); 31.6

(C-2, t); 29.2 (C-25, q); 28.2 (C-16, t); 26.1 (C-23, t); 24.3 (C-15, q); 23.0 (C-28, t); 21.0 (C-11, t); 19.8 (C-27, q); 19.3 (C-19, q); 19.0 (C-26, q); 18.7 (C-21, q); 11.8 (C-18, q); 11.9 (C-29, q).

Analysis calculated for $C_{29}H_{50}O$: C, 83.99; H, 12.15. Found: C, 83.16; H, 12.42.

Phytoecdysteroids

Ecdysterone & 2β, 3β, 22α-Triacetate

(22R)-2β,3β,14α,20β,22α,25-hexahydroxycholest-7-en-6-one, m.p.: 237-240 ° C (Lit. 240 ° C, Takemoto *et al.* 1967).

^{1}H NMR ($CDCl_3$, Triacetate, δ): 5.85 (1H, s, H-7); 5.31 (1H, br s, H-3α); 5.04 (1H, dt=9, 3 Hz, H-2α); 4.79 (1H, d=9 Hz, H-22β); 3.10 (1H, br t=7.8 Hz, H-9α); 2.10 (6H, s, 2X OAc); 1.99 (3H, s, OAc); 1.26 (3H, s, Me-21); 1.23 (3H, s, Me-26*); 1.21 (3H, s, C-27); 1.04 (3H, s, Me-19); 0.85 (3H, s, Me-18).

^{13}C NMR ($CDCl_3$, Triacetate, δ): 202.1 (C-6); 172.2 (OAc); 170.3 (OAc); 170.0 (OAc); 164.8 (C-8); 121.6 (C-7); 84.4 (C-14); 79.8 (C-22); 77.0 (C-20); 70.4 (C-25); 68.7 (C-3); 67.2 (C-2); 51.0 (C-5); 49.6 (C-17); 47.6 (C-13); 40.4 (C-24); 38.4 (C-1*); 38.3 (C-10*); 34.1 (C-4); 33.7 (C-9); 31.5 (C-15); 31.2 (C-12); 30.2 (C-27); 29.2 (C-16); 28.5 (C-26); 24.7 (C-23); 23.8 (C-19); 20.6 (C-21†); 20.5 (C-11†); 17.5 (C-18).

IR, *v* max (KBr, cm^{-1}): : 3400, 2960, 2870, 1643, 1450, 1370, 1050, 880.

UV max (ethanol): 243 nm (ε 10,400)

Analysis calculated for $C_{27}H_{44}O_7$: C, 67.47; H, 9.23. Found: C, 67.08; H, 9.30.

Ponasterone A and 2β, 3β, 22α Triacetate

(22R)-2β, 3β, 14α, 20β, 22α-pentahydroxycholest-7-en-6-one. M.p. : 256-262 ° C (Lit. 259-260 ° C, Nakanishi *et al.* 1966)

^{1}H NMR ($CDCl_3$, Triacetate, δ): 5.87 (1H, s, H-7); 5.31 (1H, br s, H-3α); 5.04 (1H, dt=9, 3 Hz, H-2α); 4.84 (1H, d=9 Hz, H-22β); 3.12 (1H, br t=7.8 Hz, H-9α); 2.11 (6H, s, 2X OAc); 2.01 (3H, s, OAc); 1.25 (3H, s, Me-21); 1.03 (3H, s, Me-19); 0.89 (3H, d=2.7 Hz, Me-26*); 0.87 (3H, d=2.7 Hz, Me-27*); 0.85 (3H, s, Me-18).

^{13}C NMR ($CDCl_3$, Triacetate, δ): 201.9 (C-6); 172.3 (OAc); 170.4 (OAc); 170.1 (OAc); 164.7 (C-8); 121.5 (C-7); 84.4 (C-14); 79.5 (C-22); 76.9 (C-20); 68.7 (C-3); 67.1 (C-2); 50.9 (C-5); 49.6 (C-17); 47.5 (C-13); 38.3 (C-1); 35.7 (C-10); 34.1 (C-4); 33.6 (C-9); 31.5 (C-15); 31.2 (C-12); 29.2 (C-16); 27.9 (C-23); 27.7 (C-25); 23.8 (C-21); 22.9 (C-19); 22.1, 21.1 (C-27*); 21.0 (C-26*); 20.6 (C-24†); 20.5 (C-11†); 17.4 (C-18).

IR, ν max (KBr, cm^{-1}) : 3400, 2960, 2870, 1643, 1450, 1380, 1050, 870.

Analysis calculated for $C_{27}H_{44}O_6$: C, 69.79; H, 9.54. Found: C, 69.41; H, 9.72.

Phenolic Compounds

Usnic Acid

m.p. : 208-213° C, yellow orthorhombic prisms from ligroin/ethyl acetate (lit. 204, acetone, Schopf & Ross, 1938). $[\alpha]_D^{25}$ -510, $CHCl_3$, c 0.62 (lit. –509, $CHCl_3$, c 0.679, Schopf & Ross, 1938).

^{1}H NMR ($CDCl_3$, 300 MHz, δ): 1.76 (3H, s, C-4' angular methyl); 2.10 (3H, s, C-6 aromatic methyl); 2.67 (6H, s, C-3 & C-8 acetyl methyls); 5.97 (1H, s, C-1); 11.02 (1H, s, C-5 phenol); 13.30 (1H, s, C-7 phenol); 18.83 (1H, s, C-4 enol).

^{13}C NMR ($CDCl_3$, 75 MHz, δ): 7.5 (q, C-6 methyl); 27.9 (q, C-4' angular methyl); 31.3 (q, C-8 acetyl methyl); 32.1 (q, C-3 acetyl methyl); 59.0 (s, C-4'); 98.3 (d, C-1); 101.5 (s, C-3); 103.9 (s, C-8); 105.2 (s, C-6); 109.2 (s, C-5'); 155.1 (s, C-8'); 157.4 (s, C-7); 163.8 (s, C-5); 179.0 (s, C-1'); 191.6 (s, C-8 acetyl CO); 198.0 (s, C-2 CO); 200.3 (s, C-3 acetyl CO); 201.7 (s, C-4 CO).

IR, ν max (KBr, cm^{-1}): 1690 (dienone carbonyl at C-2); 1630 (aromatic C-acetyl); 1610 (enol ether and aromatic double bonds); 1540 (conjugate carbonyl at C-3).

Analysis calculated for $C_{18}H_{16}O_7$: C, 62.77; H, 4.69. Found: C, 62.47; H, 4.75.

Betuloside (4-(4'-Hydroxyphenyl)-2R-butanol Glucoside)) & Aglycone

M.p. : 191-193 ^{o} (Lit. 187-190 ^{o} Khan *et al.* 1976).

^{1}H NMR ($CDCl_3$, δ): 1.17 (3H, d, Me, J=5.7 Hz); 1.8 (2H, cm, H-3); 2.58 (2H, t, H-4, J=7.5 Hz); 3.6-4.5 (glucosyl); 6.8 & 7.0 (4H, d, A_2B_2 Aromatic, J=8.2 Hz); 8.84 (1H, s, 4'-phenol).

^{13}C NMR ($CDCl_3$, aglycone, δ): 153.9 (C-4'); 133.7 (C-1'); 129.4 (C-2', 6'); 115.3 (C-3', 5'); 67.8 (C-2); 40.7 (C-3); 31.2 (C-4); 23.4 (C-1 Me).

IR, ν max (KBr, cm^{-1}): 3370, 2930, 2860, 1610, 1590, 1510, 1435, 1445, 1370, 1230.

Analysis calculated for $C_{10}H_{14}O_2$: C, 72.26; H, 8.49. Found: C, 72.12; H, 8.56.

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BIOGRAPHICAL SKETCH

Richard Michael Davies was born in Cocoa, Florida on March 21, 1959 to Dan and Ruth Lewis Davies. He attended Rockledge High School where he participated in the cross country and track teams, the school's concert and marching bands, and other extracurricular activities and societies. He attended Brevard Community College for one year before enrolling at the University of Florida to complete bachelor's degrees in chemistry and then pharmacy. While studying pharmacy he also worked on projects with Professor Rao in the laboratory. Interest in the chemistry and research of natural products and the development of new anticancer therapies brought him back for graduate studies.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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